Univerzita Karlova

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Biochemie

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The effect of antimicrobial peptides on *Saccharomyces cerevisiae* and other yeast species
Studium účinku antimikrobiálních peptidů na *Saccharomyces cerevisiae* a další druhy kvasinek

Diploma thesis
Diplomová práce

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Conzultants: RNDr. Hana Sychrová, DrSc., RNDr. Marie Kodedová, Ph.D.

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Here I declare, that I wrote this master thesis on my own, under the guidance of supervisor Prof. RNDr. Petr Hodek, CSc., and consultants RNDr. Hana Sychrová, DrSc., RNDr. Marie Kodedová, Ph.D., and I correctly cited all the used sources.

Prohlašuji, že jsem tuto diplomovou práci vypracovala samostatně pod vedením školitele Prof. RNDr. Petra Hodka, CSc. a konzultantek RNDr. Hany Sychrové, DrSc. a RNDr. Marie Kodedové, Ph.D. a všechny použité prameny jsem řádně citovala.

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Abstract

The increased use of antibiotics, antifungal agents and disinfectants in the last decades has resulted in development of microbial resistance to these drugs. *Candida* species are the fourth most common cause of hospital-acquired bloodstream infection and kill 40% of those patients. Natural antimicrobial peptides are promising candidates for the development of new agents to treat yeast and bacterial infections, as their presumed mechanism of action differs significantly from the mechanism of action of current drugs. This work is focused on several peptides isolated from the venom of wild bees and their synthetic analogues and the identification of the most effective ones against non-pathogenic *Saccharomyces cerevisiae* and several pathogenic *Candida* species.

Antifungal activity of eight cationic antimicrobial peptides was tested and compared under various conditions. The overall susceptibility of pathogenic yeast species to currently used antifungal drugs and the antimicrobial peptides was screened with the aim to identify potential synergistic and species-specific effects.

The effect of antimicrobial peptides on membrane potential was measured by a fluorescent probe (diS-C_3(3)), and the relative hyperpolarization of plasma membrane was shown for each peptide. The effect of antimicrobial peptides on yeast viability was established, depending on specific conditions, such as concentration of the peptides, pH or concentration of ions in the environment. In addition, the combination of antimicrobial peptides with conventional antifungal drugs was tested, and positive or negative effect of various combinations and different ratios of concentrations of the peptides to the drugs were demonstrated.

As an advanced level of testing of antimicrobial peptides, *in vivo* system was used to assess the efficiency under an interaction with an immune system. Larvae of *Galleria mellonella* were infected by the most drug-resistant yeast strains and treated by one selected peptide acting the best in the previous experiments.

Key words

Antimicrobial peptides, *Candida* infection, antifungal activity, yeast growth, membrane potential, *Galleria mellonella*

Abstrakt

Z důvodu nadměrného užívání antibiotik, antimykotik a dezinfekčních prostředků v posledních desetiletích, byla u patogenních mikroorganismů vyvinuta značná rezistence vůči těmto látkám. Kvasinky rodu *Candida* představují čtvrtého nejčastějšího původce kvasinkových infekcí u hospitalizovaných pacientů a pro 40% z nich jsou letální. Díky mechanismu účinku rozdílným od zmíněných současně užívaných antimykotik a antibiotik se antimikrobiální peptidy stávají slibnými kandidáty pro vývoj nových léčiv proti kvasinkovým a bakteriálním infekcím. Tato práce se zabývá peptidy izolovanými z divokých včel a jejich syntetickými analogy a identifikuje ty nejefektivnější v boji proti nejčastějším druhům kvasinek *Candida* a modelové kvasince *Saccharomyces cerevisiae*.

Byl testován a porovnáván antifungální efekt osmi peptidů v různých definovaných podmínkách. Za účelem identifikace případného synergistického působení antimikrobiálních peptidů a současně užívaných antimykotik byla zjišťována citlivost patogenních kvasinek k těmto konvenčním antifungálním látkám, peptidům, a k jejich kombinacím. Odpověď kvasinkových buněk na úrovni plasmatické membrány byla studována měřením relativního membránového potenciálu pomocí fluorescenční sondy (diS-C_3(3)). Hyperpolarizační efekt byl pozorován pro každý jednotlivý peptid.

Dále byla sledována aktivita antimikrobiálních peptid v přežívání kvasinkových buněk a vliv faktorů jako pH či koncentrace iontů v daném prostředí.

Pro vyšší úroveň testování *in vivo* byl zvolen modelový organismus *Galleria mellonella*, ukazující efektivitu peptidů při rozvoji kvasinkové infekce v tomto hostitelském organismu.

Klíčová slova

Antimikrobiální peptidy, *Candida*, infekce, antifungální aktivita, růst kvasinkových buněk, membránový potenciál, *Galleria mellonella*

Abbreviations

5-F-dUMP	5-fluorodeoxyuridine monophosphate
aa	Amino acid
ABC	ATP-binding cassette
AMPs	Antimicrobial peptides
ATP	Adenosin triphosphate
CTG	CTG codon
diS-C_3(3)	3,3'-dipropylthiacarbocyanine iodide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleosidtriphosphates
EDTA	Ethylenediaminetetraacetic acid
hBD	Human β-defensins
LPS	Lipopolysaccharide
MDR	Multidrug resistance
MES	2-(N-morpholino)ethanesulfonic acid
MFS	Major facilitator superfamily
NADPH	Nicotinamide adenine dinucleotide phosphate
NQO	4-nitroquinoline N-oxide
PBS	Phosphate-buffered saline
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
UC	Universal classification system
UCBB	Circular polypeptides with a peptide bond
UCLL	Linear one-chain or two linear peptides
UCSB	Polypeptide chains with a sidechain
UCSS	Sidechain-sidechain linked peptides
UMP	Uridine monophosphate
UTP	Uridine triphosphate

WGD	Whole genome duplication
YNB	Yeast nitrogen base
YPD	Yeast extract peptone dextrose

Contents

1. Introduction

1. 1 Yeasts

Yeasts are unicellular fungi, widely spread in natural habitats. Yeasts commonly appear on plants, in salt and fresh water or soil, and are found on epithelial surfaces and in the gastrointestinal tracts of animals, where they can live symbiotically or parasitically. The overall constitution of the yeast cell is eukaryotic. It is separated from the surrounding environment by cell wall and plasma membrane, and it contains number of organelles, such as nucleus separated by double membrane connected with endoplasmic reticulum, Golgi complex, mitochondria and vacuoles. Buds are typical for many yeast species while dividing. The particular cell components are shown in Fig. 1.

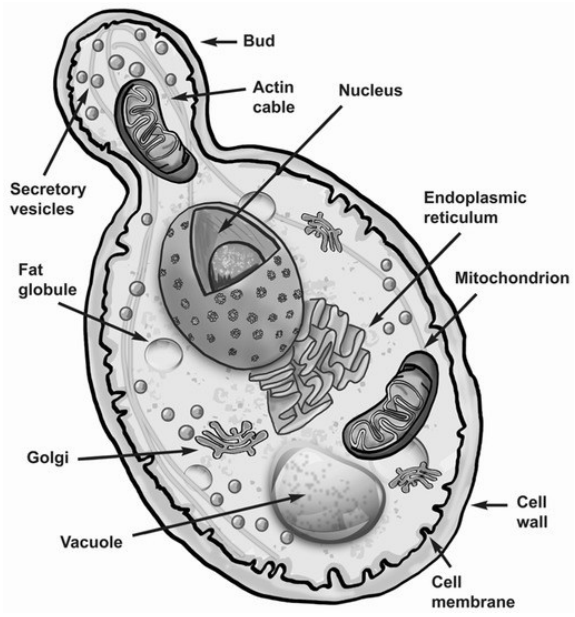


Fig. 1. Schematic picture of a yeast cell. The characteristic components are cell wall, cell membrane, nucleus, endoplasmic reticulum, Golgi apparatus, mitochondria, vacuoles and secretory vesicles [1].

1.1.1 *Saccharomyces cerevisiae* as a model organism

Yeast, as a model organism, brings some key advantages in research approaches, such as easy culture manipulation and the relative simplicity in cell constitution while belonging to the complex eukaryotic organisms' clade. A big milestone in molecular biology and genetics

came with releasing the whole sequence of *Saccharomyces cerevisiae* genome, the main representative of yeasts [2]. Most of the genes of higher eukaryotes, including human, have their homologues within the yeast genome. The complete yeast genome sequence is extremely useful as a reference for gene function or regulation analysis. In addition, the comfort genetic manipulation of yeast allows an easier way in investigation of particular gene products, compared to work with other eukaryotes. The importance of yeast genetics comes from the ability to simply map some of the phenotype-causing gene to a region of the yeast genome. For the past several decades *S. cerevisiae* has been the ideal model organism for molecular genetic experiments as the basic cellular mechanisms of metabolism, replication, recombination, cell division and gene expression are generally conserved in yeast and higher eukaryotes [3].

The best known and commercially important yeast is *Saccharomyces cerevisiae*. This non-pathogenic organism is broadly used as baker's yeast and for fermentations. This species is used to ferment the sugars of grapes, wheat, rice or corn to produce alcoholic beverages and in the baking industry to enlarge or shape up dough. It is also taken as a vitamin supplement due to its composition of 50 % protein and rich source of vitamins B, folic acid and niacin [4].

Genomic analysis revealed the phylogenetic relationships between Saccharomycotina subphylum. The most important groups are the WGD (‘whole genome duplication’) within Saccharomycetaceae clade and CTG *Candida* clade, which translates CTG codons as serine instead of leucine [5]. The phylogenetic relationships between the species used in this work are shown in Fig. 2.

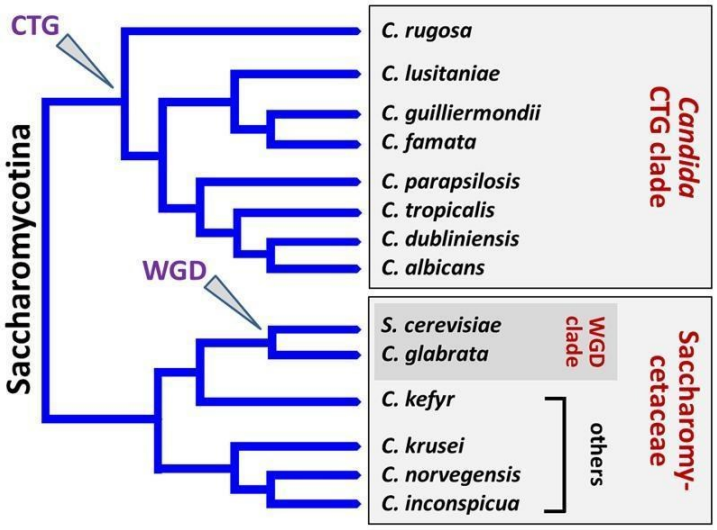


Fig. 2. *C. albicans, C. dubliniensis*, *C. tropicalis* and *C. parapsilosis* form part of the *Candida* CTG clade. Meanwhile, *C. glabrata* with *S. cerevisiae* belong to the "whole genome duplication" (WGD) clade within Saccharomycetaceae. The remaining species used in this work *C. krusei* is probably closely related to the Saccharomycetaceae clade. The branch lengths are arbitrary [5].

1.1.2 *Candida* species

Candida genus contains around 200 species and tens of them are opportunistic human pathogens [6]. Normally, they inhabit the skin, mouth, intestinal tract and vagina without harming the human host. However, children, hospitalized patients or those treated by broad-spectral antibiotics and immunocompromised people (for example, undergoing transplantation, chemotherapy or suffering from AIDS) have problems with maintaining the symbiotic life, which results in excessive growth of yeasts [7].

The most common yeast infection is candidiasis caused by *Candida albicans*. Usually, it causes diaper rash and thrush of the mouth and throat or vaginal infection, so-called vulvovaginitis. According to their virulence, these infections can be treated by available antifungals and are not dangerous for the patient. Nevertheless, the infection becomes life-threatening at the point when the pathogenic yeast reaches the bloodstream and major body organs and causes a systemic disease, called candidemia, which generates around 85% of all invasive fungal infections [8]. In this case, the mortality achieves up to 40% of all infected patients. The vast majority of candidemias are caused by a few other species, which are investigated in this work.

The most widespread is *C. albicans*, but its relative incidence is decreasing, while the prevalence of other species is increasing. Since the 1980s, remarkable expansion of infections due to non-*albicans Candida* species was observed, especially *C. glabrata*, *C. parapsilosis* and *C. tropicalis*, followed by *C. krusei*. Rarely, the infections are evoked by *C. dubliniensis*. The representative ratios vary between the particular geographic localization [9].

Pictures of *Candida* species mentioned above taken by light microscope using 100 × 10 magnification are shown in Fig. 3.

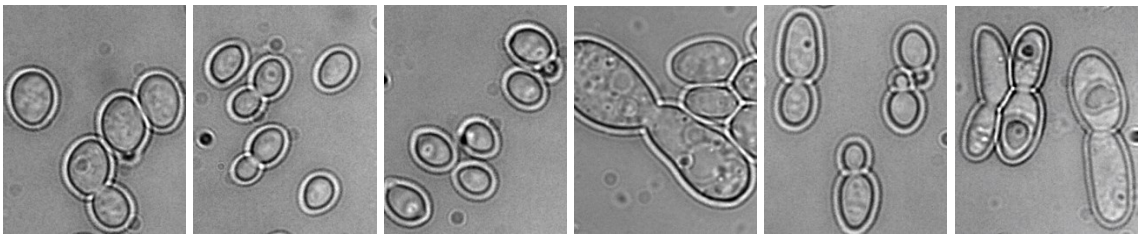


Fig. 3. Photographs of representative *Candida* species taken by light microscope. *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis, C. dubliniensis and C. krusei*. The pictures were taken with immerse oil with the same magnification 100×10.

1.1.2.1 *Candida albicans*

C. albicans is the primary commensal species, as well the one causing candidiasis and candidemias [10]. This species differs from the others especially with its significant morphological polymorphism, which is its characteristic pathogenic feature. There are several distinct cell forms described, as hyphae, varying in color, shape or cell wall thickness. *C. albicans* changes the particular forms depending on pH, temperature and stage of the disease [11]. *C. albicans* possesses also other pathogenic mechanisms, including adhesion by expression of adhesins, which bind to surface proteins of human cells, invasion of host cells by expression of invasins which induce endocytosis, or secretion of fungal hydrolases which allow penetration to host cells [12]. Common phenomenon is biofilm formation. The biofilm structure is composed of lower and upper layer of cells and the extracellular matrix and provides protection against antifungal agents. Biofilm occurs especially at abiotic surfaces, such as catheters, in hospital environments [13].

1.1.2.2 *Candida glabrata*

This species is a common pathogen especially in the United States [9]. In the patients with oral candidiasis, the most frequent cause is mixed species infection by combination of *Candida* species *C. glabrata* and *C. albicans* [14]. The incidence is higher in adults than in kids or neonates [15]. *C glabrata* is also capable to form biofilms. Cells of *C. glabrata* are noticeably smaller (1 – 4 μm) than the yeast cells of *C. albicans* (4 – 6 μm), *C. tropicalis* (4 – 8 μm) and *C. parapsilosis* (2.5 – 4 μm) [16]. *C. glabrata* is not polymorphic, lacking the ability to form hyphae, growing only in yeast form. An important characteristic of *C. glabrata* is its haploid genome, in contrast to the diploid genome of most of the other *Candida* species, and its very close relationship to *S. cerevisiae* [17].

1.1.2.3 *Candida parapsilosis*

Candidemia caused by *C. parapsilosis* is commonly diagnosed in the United Kingdom, causing more than a quarter of all systemic fungal infections in low-birth-weight infants and in North America, causing up to one-third of neonatal *Candida* invasive infections [9]. Additionally, it is the major fungal organism isolated in neonatal intensive care units, where it is linked to neonatal mortality around 10 % [9]. Unlike *C. albicans* or *C. tropicalis*, which appear in multiple morphologic formations, *C. parapsilosis* does not form true hyphae but forms either a yeast phase or a pseudohyphae, which is large and curved, it is referred to as 'giant cells'. *C. parapsilosis* is notorious for its ability to grow in parenteral nutrition and to form biofilms on implanted devices and for persistence in the hospital environment [18]. However, *C. parapsilosis* systemic infection displays overall a lower mortality rate than *C. albicans* and *C. glabrata* [19].

1.1.2.4 *Candida tropicalis*

This species is commonly identified especially in Brazil and the rest of Latin America, but also in Europe or the USA [9]. *C. tropicali*s is often associated with patients suffering from malignancy and neutropenia [20]. The typical property is relative high mortality and dissemination when compared with other non-*albicans* infections. This characteristic can be connected with the virulence factors such as biofilm formation, proteinases secretion and dimorphism [17].

1.1.2.5 *Candida dubliniensis*

C. dubliniensis was first isolated from the oral cavity of HIV infected patients in Dublin [21]. Unlike other *Candida* commensals, this species does not normally inhabit the gastrointestinal tract of humans. In contrast, it primarily appears in the oral carriage and oropharyngeal part of HIV-positive and AIDS patients [22]. *C. dubliniensis* is polymorphic, forming hyphae, pseudohyphae and also chlamydospores [23].

1.1.2.6 *Candida krusei*

This species causes just 2 — 4 % of reported candidemia [9]. However, it is notorious for its natural fluconazole resistance, which evolves from a different structure of the target enzyme 14α-demethylase [24]. Especially, patients with bone marrow or blood transplants belong to the significant group afflicted by *C. krusei* [25].

1.2 Conventional antifungal drugs in clinical practice

For fighting local or systemic fungal infections, fungicidal and fungistatic drugs are used. Since both the yeast and the host cells are eukaryotic, the most challenging part of the fungal infection eradication is the selectivity of the process. The antifungals used nowadays mostly target the cell surface, specifically the components of fungal plasma membrane or the cell wall and its biosynthetic pathways.

Sterols are important components of plasma membranes, influencing especially the membrane fluidity, asymmetry and proteins anchoring. In animal cells, the main sterol is cholesterol, while ergosterol is the predominant sterol in yeast cells. This difference between human and fungal cell membranes is exploited by most of the current antifungals either by directly targeting the ergosterol or by blocking the enzymes of its synthesis [26]. There are seven major chemical classes of antifungals: azoles, polyenes, pyrimidine analogues, echinocandins, allylamines, thiocarbamates and morpholines. The groups targeting the different steps of ergosterol synthesis are schematically shown in Fig. 4, and described deeper in this chapter together with antifungals targeting the cell wall or acting intracellularly.

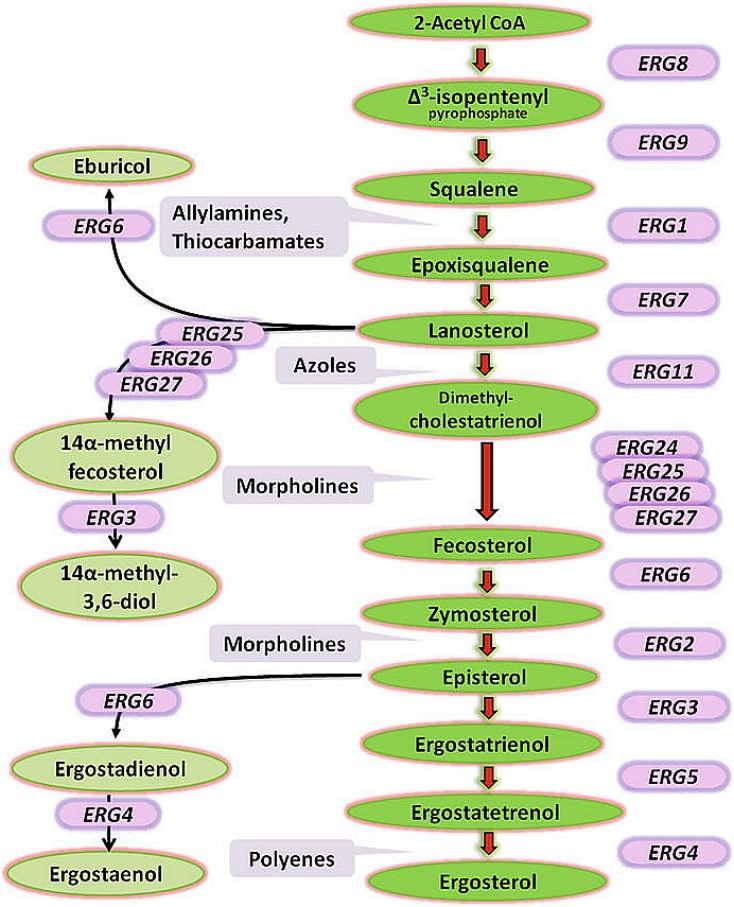


Fig.4. Ergosterol biosynthesis pathway showing specific point of action of antifungal drugs. Different classes of antifungal drugs are shown on left against the steps of their action in pathway with corresponding enzymes catalysing the reaction steps shown on right. Taken from [24].

1.2.1 Azoles

The fungistatic azoles act by targeting lanosterol 14α-demethylase encoded by *ERG11* gene causing the blocking of cytochrome P450-dependent conversion of lanosterol to ergosterol. The inhibition of 14α-demethylase leads to an accumulation of toxic methylated sterols resulting in the membrane stability stress and its distraction. The lack of ergosterol can lead to dysfunctions in cell growth and proliferation of inhibition. In addition, an interference with NADPH-dependent 3-ketosteroid reductase encoded by *ERG27*, catalysing the last step in 14α-methyl fecosterol biosynthesis, was observed, which results in toxic accumulation of

obtusifolione [27]. Taken all together, the mechanism of action of azoles is complex, involving more factors.

There are two main subclasses distinguished according to the number of nitrogen atoms comprised in the azole ring. Ketoconazole, clotrimazole, miconazole, oxiconazole or econazole contain two nitrogen atoms in the ring, therefore forming imidazoles. Fluconazole, itraconazole, posaconazole, terconazole and voriconazole are triazoles [28]. Imidazoles are generally applied against the mucosal fungal infections while triazoles are used both for the systemic as well as for the mucosal infections [29]. The chemical structures of azoles used in this work are shown in Fig. 5.

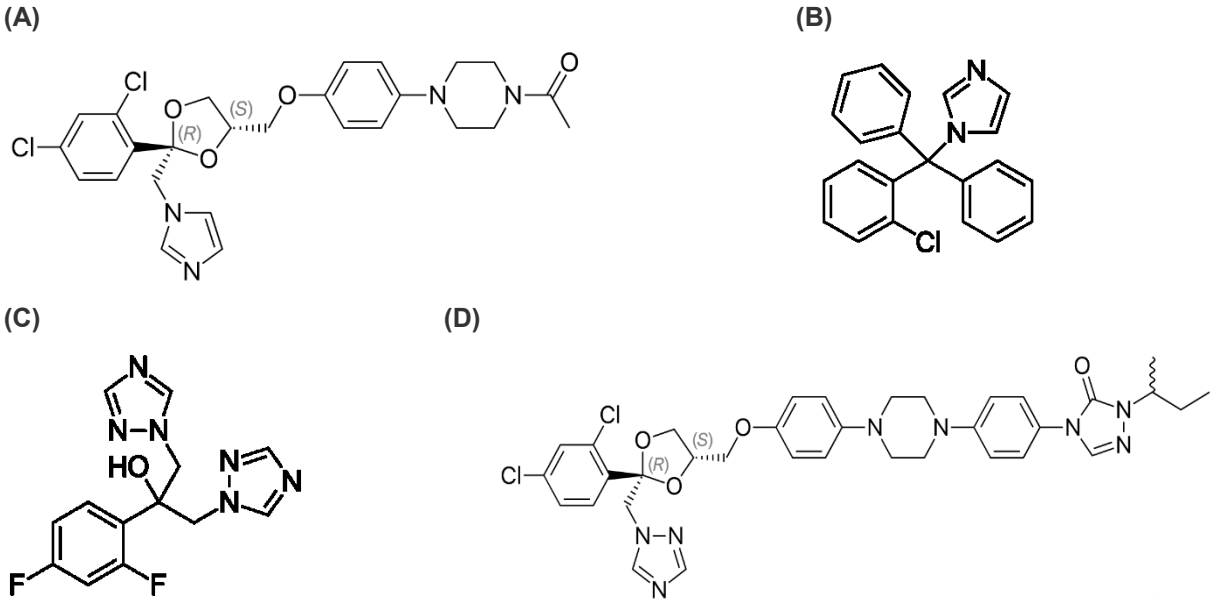


Fig. 5. Chemical structures of representative azoles. Imidazoles are represented by ketoconazole (A) and clotrimazole (B). Triazoles include fluconazole (C) and itraconazole (D). Downloaded from wikipedia.com. (10.10.2017)

1.2.2 Polyenes

These amphipathic molecules which are subclass of so-called macrolides are naturally produced by the bacterial genus *Streptomyces* [30]. Polyenes target directly the ergosterol in the yeast membranes. They are supposed to bind hydrophobically in the manner of forming

pores in the membrane, which discards the vital permeability, alter the electrochemical gradient, cause release of intracellular fluids and results in the cell death [31].

The most famous example of polyenes used for the treatment of systemic yeast infections is amphotericin B, its chemical structure is shown in Fig. 6. Others like natamycin and nystatin are preferentially applied to the treatment of topical infections. A remarkable advantage of amphotericin B is its fungicidal character and lower frequency of resistance occurrence. On the other hand, there is a significant problem with its usage, because of its severe side effects, especially nephrotoxicity [32].

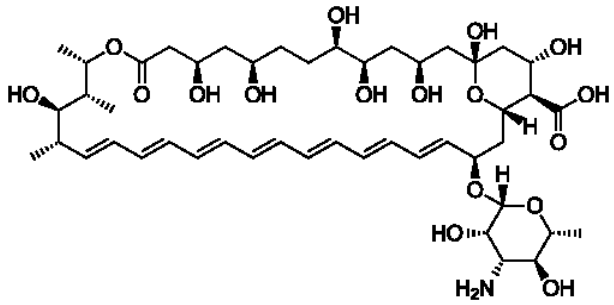


Fig. 6. The chemical structure of amphotericin B. Downloaded from wikipedia.com. (10.10.2017)

1.2.3 Allylamines, thiocarbamates and morpholines

Allylamines and thiocarbamates inhibit the early stage of ergosterol biosynthesis, the squalene epoxidase encoded by *ERG1* gene, which leads to accumulation of squalene in membrane, which disrupts the membrane function [33]. Morpholines inhibit the *ERG24* and *ERG2* gene products of ergosterol biosynthesis and include amorolfine used in medicine and fenpropimorph used in agriculture. An example of thiocarbamates is tolnaftate. Allylamines include terbinafine, its chemical structure is shown in Fig. 7. Above-mentioned drugs are applied for the control of dermatophyte infections [26].

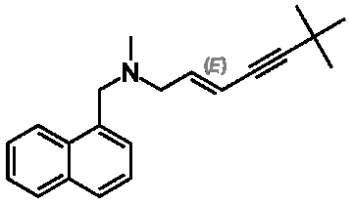


Fig. 7. The chemical structure of terbinafine (E = cis isomery). Downloaded from wikipedia.com. (10.10.2017).

1.2.4 Pyrimidine analogues

The representative of the group of intracellularly acting substances is 5-fluorocytosine. Its chemical structure is shown in Fig. 8. As soon as it enters the cell by a pyrimidine-specific permease, intracellular cytosine deaminase converts the molecule into 5-fluorouracil, which afterwards replaces the physiological uridine monophosphate (UMP) and uridine triphosphate (UTP) precursors of RNA molecules. The modified uracil incorporated to the RNA disables the proper proteosynthesis. In addition, 5-fluorocytosine interferes with nuclear division via converting to 5-fluorodeoxyuridine monophosphate (5-F-dUMP), an inhibitor of thymidylate synthase, which is involved in DNA replication [34].

Due to a relatively frequent development of tolerance to 5-fluorocytosine by the yeasts, this compound is usually used in combination with other antifungal agents to improve their efficiency [26].

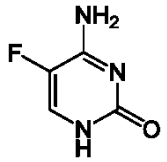


Fig. 8. The chemical structure of 5-fluorocytosine. Downloaded from wikipedia.com. (10.10.2017)

1.2.5 Inhibitors of glucan synthesis

Echinocandins, aculeacins and papulacandins target the glucan synthesis of the cell wall by noncompetitive inhibition of the β-(1,3)-D-glucan synthetase. Generally, β-glucans are specific components of yeast cell wall. Therefore, as a result of the cell wall dysfunction, the cells become susceptible to osmotic stress, grow as pseudohyphae, newly grown buds are unable to separate from mother cells which leads to cell lysis [35].

1.3 Drug resistance in yeast

Clinical multidrug resistance is a multifactorial phenomenon, which is achieved by several strategies which contribute to reduced susceptibility to not just single drug but also show collateral resistance to numerous drugs, what significantly influences the clinical impact.

Primary resistance to antimicrobial agents means that an organism is naturally invulnerable to a particular drug treatment. An example is the primary resistance of *C. krusei* to azoles due to the different structure of target lanosterol 14α-demethylase, which cannot be inhibited by azoles [36].

Especially fungistatic drugs bring the threat of development of secondary acquired resistance towards them. Different strategies of resistance to antifungals have been evolved, including mutations in drug target or its overexpression, reduction of intracellular drug concentration by its efflux or possessing entry barriers, alteration in sterol biosynthesis [37]. The known resistance mechanisms are schematically shown in Fig. 9.

A very common strategy of acquired antimicrobial resistance is the intracellular drug concentration reduction as a result of activity of MDR (multidrug resistance) transporters. The resistant cells do not accumulate the required effective drug concentration inside the cells as a consequence of upregulation of expression of multidrug efflux transporter genes. There are two main families of MDR pumps, which differ in the mechanism of transport and the used driving force.

One family contains ATP-binding cassette (ABC) transporters, which work as ATPases. ABC transporters are built of two membrane domains, each of them containing usually 6 segments interacting with the drug substrates and two intracellular nucleotide-binding domains responsible for hydrolysis of ATP, which is needed to power the efflux [38]. Especially, ABC transporters *Ca*Cdr1 and *Ca*Cdr2 of *C. albicans*, homologous *Cg*Cdr1 and *Cg*Pdh1 of *C. glabrata* or *Cd*Cdr1 of *C. dubliniensis* are the best known ones [39].

The second group belongs to the major facilitator superfamily (MFS) transporters. MFS transporters contain either 12 or 14 transmembrane segments and function as H^+ ions antiporters without the requirement of ATP [40]. Among these transporters, it is the

overexpression of Mdr1 in *C. albicans*, *C. glabrata* and *C. dubliniensis*, which contributes the most to the drug resistance [40].

Another difference between ABC and MFS transporters is the specificity towards azoles. In *C. albicans*, almost the entire spectrum of azole antifungals are substrates for ABC transporters, while MFS transporters accept exclusively fluconazole as a substrate [40].

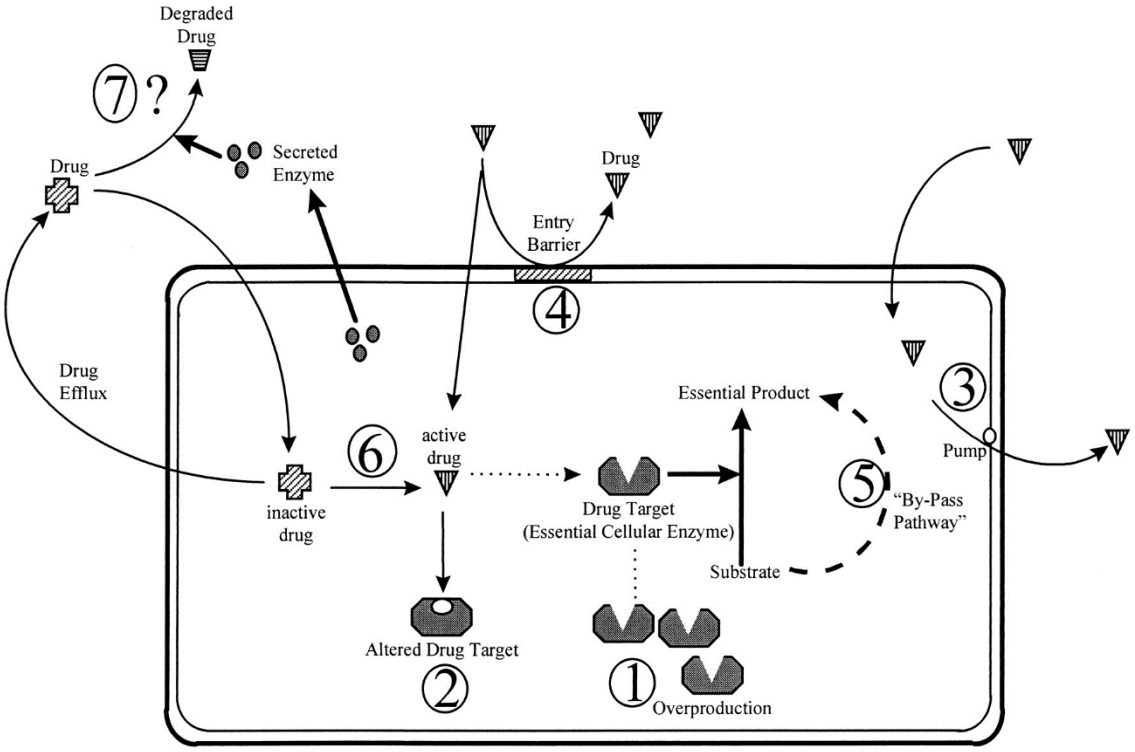


Fig. 9. Mechanisms which yeast cells might develop resistance by. 1) The target enzyme is overproduced, so that the drug does not inhibit the biochemical reaction completely. 2) The drug target is altered so that the drug cannot bind to it. 3) The drug is pumped out by an efflux pump. 4) The entry of the drug is prevented at the cell membrane/cell wall levels. 5) The cell has a bypass pathway that compensates for the loss-of-function inhibition due to the drug activity. 6) Some yeast "enzymes" that convert an inactive drug to its active form are inhibited. 7) The cell secretes some enzymes to the extracellular medium, which degrade the drug. Taken from [36].

Due to the indiscriminate use of antibiotics and antifungals in the last decades, the pathogenic microorganisms, exposed to those agents, have generated an elevated level of drug resistance. Obtaining novel antimicrobial compounds have not kept pace with the development of tolerance or resistance to available drugs. Therefore, the demand for development of novel

drug candidates has been increasing rapidly. This public health problem needs a solution in searching therapeutic agents with an antimicrobial activity, which is different from the traditional antibiotics and antifungals to prevent the risk of acquired resistance developing in their target pathogen populations. In this field, antimicrobial peptides have a potential to be used as the drug agents meeting those most important requirements.

1.4 Antimicrobial peptides

Antimicrobial peptides (AMPs) are molecules primarily acting against microorganisms. These evolutionary ancient peptides are gene-encoded and ribosomally synthesized, taking part in the innate defense system of all classes of living organisms [41]. Endogenous AMPs are ubiquitous in the nonspecific immune response of prokaryotes and eukaryotes as well. The expression of AMPs can be constitutive or inducible by infectious/inflammatory stimuli, like cytokines, bacteria, fungi, and so on. These molecules, alsoreferred to as host defense peptides, are essential not only to eliminate broad spectrum of invading pathogens urgently but also additionally to further clean up the system [42].

The discovery of AMPs started in 1939, when a bactericidal agent from a soil *Bacillus* strain, acting against pneumococcal infection, was extracted and later identified and named as gramicidin [43]. More massive investigation of AMPs began after the discovery of cecropin, which was isolated from the moth pupae of *Hyalophora cecropia* in 1980 [44]. In response to the need of new antimicrobials, more than 2000 AMPs have been characterized so far. The Antimicrobial Peptide Database was founded in 2004, as the most complete list of described AMPs [43].

1.4.1 General properties of AMPs

AMPs are a very diverse group, so it is not trivial to generalize their properties. But it is possible to point out some most significant features.

First feature is the size; AMPs are relatively small, composed maximally of tens of amino acids, while the sequence is very variable without an obvious homology. There are ultra-small (2 – 10 aa), small (10 – 24 aa), medium (25 – 50 aa) or large (50 – 100 aa) AMPs [45]. The relative representation of the length groups is shown in Fig. 10A. The length is

crucial for the effect because at least around 7 amino acids are necessary to form amphipathic structures. To transverse a lipid bilayer by α-helix, at least 22 amino acids are needed and by β-sheet about 8 amino acids long peptides are necessary. It was shown that the length of AMPs affects the cytotoxicity to the host cells, especially the hemolytic activity, so the size has to be considered to lower the problem of cytotoxicity for erythrocytes [46].

Second feature is the net charge, which is the main factor of the initial interaction with a cell surface. Most of AMPs are basic, which means cationic in neutral pH (their isoelectric points are between 8.9 – 10.7; Fig. 10B). Nevertheless, there are also neutral or anionic AMPs known [47].

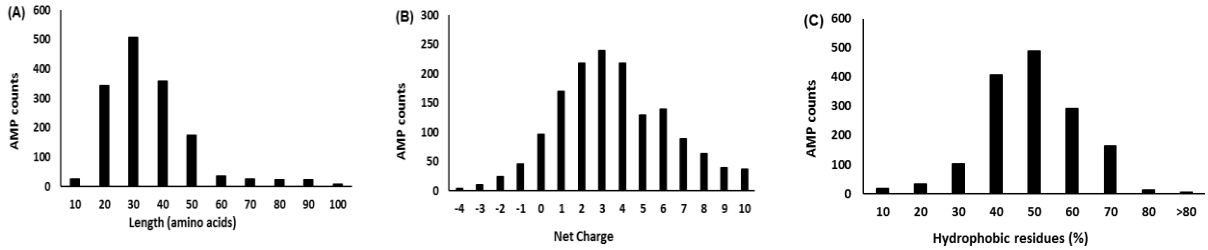


Fig. 10. Numbers of known AMPs ordered according to their (A) length, (B) net charge and (C) hydrophobicity. Modified according to [48].

Third feature is the conformation. Most of AMPs are able to fold in amphipathic conformations in membranes or membrane mimicking environment. The amphipathicity is required for making a strong partition into the membrane interface.

According to the ratio of hydrophobic amino-acid residues in the peptide chain, the final hydrophobicity is revealed. The vast majority of AMPs contain around 50% hydrophobic residues, as seen in Fig. 10C. An increase in hydrophobicity can improve its antimicrobial activity [49], and a decrease in hydrophobicity can reduce its antimicrobial effect [50]. It was also shown that the hydrophobicity determines the range of target cells, i.e. an increasing hydrophobicity can widen the range of targets of AMPs.

1.4.2 Classification of AMPs according to their source, structure and spectrum of activity

There are many ways to classify AMPs, from different points of view [51]. First of all, they are distributed regarding the properties mentioned above: the size, the net charge and the hydrophobicity.

Another level of the diversity of the AMPs comes from the biological sources. As already mentioned, the peptides are produced by practically all organisms [52]. **Bacteria** are able to excrete bacteriocins against other potentially competitive bacterial species within the environment, which can be narrow or broad spectral AMPs. **Plant** AMPs are involved in the innate immune responses against pathogenic threads like viruses, bacteria, parasites or fungi [53]. Since **insects** have no adaptive immune system, they have developed an efficient humoral defence reaction. AMPs are synthetized in the fat body and then secreted to hemolymph [54]. There are numerous famous insect AMPs, e.g. cecropins (inducible antibacterial peptides, belonging among the first AMPs discovered), defensins (cysteine-rich polypeptides with 3 — 4 disulfide bridges, with salt-dependent antimicrobial activity, which were also isolated from plants and mammals [55]), glycine-rich AMPs (whose content of around 20% of glycine residues has an influence on tertiary structure and disrupting cell membranes) and proline-rich cationic AMPs (where proline is often associated with arginine residues in repeated motifs and exceptionally, they do not act on the membranes but intracellularly [56]). The most abundant AMPs in **animals** are α- or β-defensins and histatins.

A separate group is formed by AMPs produced recombinantly in engineered microorganisms. The recombinant methods are widely used to heterologously express the AMPs in bacteria, mostly *Escherichia coli* [57]. As most of the AMPs are toxic for prokaryotic cells, production of high levels of peptides becomes problematic. Therefore, the yeast expression system with methylotrophic *Pichia pastoris* as a host organism is used for the large-scale production of AMPs [58].

Last, but not least source of AMPs is their laboratory synthesis. Nowadays, development and synthesis of analogues of natural AMPs with high effectivity represents an

option for their use in humans. Synthetic AMPs are a new challenge in their application as novel antibiotics [59].

According to The Antimicrobial Peptide Database [43], "there is universal classification system (UC) which categorizes AMPs into four classes: UCLL – linear one-chain peptides or two linear peptides not connected via a covalent bond, UCSS – sidechain-sidechain linked peptides. A sidechain-sidechain connection can occur within a single peptide chain or between two different peptide chains, UCSB – polypeptide chains with a sidechain to backbone connection, UCBB – circular polypeptides with a peptide bond between the N- and C-termini."

There are structural forms shared by most AMPs. The structural patterns are tightly connected with mechanism of action but it should be noticed that within the different AMPs subclasses, the activity may vary considerably. According to the 3D structure, AMPs can be divided into four families. **α-helical peptides** display high cationic and amphipathic properties and act preferentially against Gram-positive bacteria and fungi, while the helical content correlates also with hemolytic activity [59]. For example, cecropins [44], magainins [61], pleurocidin [62], temporins [63], buforin [64], clavanins [65] belong to this group (Fig. 11E). **β-sheet peptides** comprise mostly antiparallel β-conformation structure often stabilized by disulphide bridges between conserved cysteine residues. They contain two or more β-sheets joined by loop (Fig. 11B) or another intramolecular turn (Fig. 11C and 11D) and can form either oligomeric transmembrane structures or aggregates on the surface of lipid bilayer [66], for example protegrin-1 [67], lactoferricin B [68] or bactenecin [69]. Another main group are human β-defensins (hBD), which are 33 – 47 long, cationic, stabilized by three intramolecular disulphide bonds [70]. They are very intensively studied [71]. Within this group, despite their low sequence conservation, the peptides possess highly homologous tertiary **mixed α/β-structure** (Fig. 11A) [72]. Some examples are hBD-1, hBD-2 [73] and hBD-3 [74]. There are also reports of AMPs with **extended structure** (Fig. 11F), which are not folded into regular structures, often rich in arginine and tryptophan residues [75] and are able to bind to heat-shock proteins, e.g. indolicin, tritrpticin, nisin, apidaecin, drosocinor and pyrrhocorcin[76] .

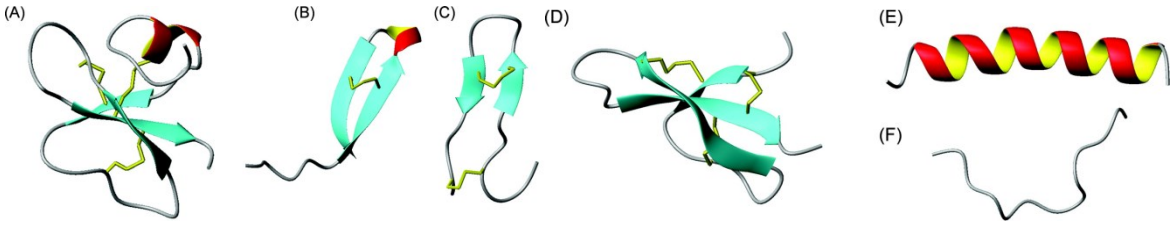


Fig. 11. Structural classes of AMPs. (A) Mixed structure of human β-defensin-2 (B) looped thanatin (C) β-sheeted polyphemusin (D) rabbit kidney defensin-1 (E) α-helical magainin-2 (F) extended indolicidin. The disulfide bonds are indicated in yellow. The figure has been prepared with the use of the graphic program MolMol 2K.1 [77].

AMPs display a wide spectrum of activities [77]. There are **antiviral AMPs**, which can act against both enveloped RNA and DNA viruses, often by integrating in the viral envelope or the host cell membrane and thus disrupting the adsorption and entry process [78]. AMPs may prevent entering the cells by binding either to the viral surface receptors, like glycoproteins [79] or receptors of host cells, like heparan sulfate [80].

Antibacterial AMPs are the best studied class to date. They can act against Gram-positive and more often Gram-negative bacteria or both. The particular mechanisms of action depend on characteristics like net positive charge, hydrophobicity and flexibility [81, 82]. AMPs are able to kill also antibiotic-resistant bacteria [83]. Besides the classical protection against bacterial infection in multicellular organisms, AMPs produced by bacteria (bacteriocins) contribute to survival of individual cells by killing other bacteria that might compete for nutrients in the environment. There are two main groups of bacteriocins distinguished: lanthionine containing (lantibiotics) and non-lanthionine containing [84, 85].

Antiparasital AMPs make up much smaller group in comparison with previous ones. There are AMPs acting against pathogens causing tropical diseases, like *Trypanosoma* [86] or *Plasmodium* or *Leishmania* [87] parasites. There are also reports about **insecticidal**, **chemotactic**, **wound healing** and **anticancer AMPs** [88].

Antifungal AMPs are the main topic of this work and are discussed in detail in chapter 1.4.4. One of the most famous groups of antifungal peptides represent so-called killer toxins, which are produced by certain yeast species in order to eliminate other potentially rival yeast species in the environment [89]. Killer toxins may cause undesired effects for example in parts of human body or in particular industrial fields, like beer brewing [90]. Cysteine-rich

peptides present an important group of antifungal peptides. They are composed of eight relatively conserved cysteine residues and four intramolecular disulfide bonds stabilizing the 3D α/β-structure. These defensins-like peptides have been isolated from many plant species [91].

There were lactoferrin-derived peptides studied for exhibiting strict homology after alignment of the lactoferrin amino-acid chains [92]. Meanwhile a "multidimensional antimicrobial signature" was defined by proteomics methods to provide an unifying stereospecific patterns shared by particular group of peptides, as a scaffold structure [93]. But later this approach turned out not to be completely universal.

1.4.3 Mode of action of AMPs

As the important effector molecules of innate immunity, AMPs are able to enhance phagocytosis, neutralize the septic effects of lipopolysaccharides, promote prostaglandin release or accumulation of various immune cells on inflammatory sites [94]. They can also stimulate angiogenesis [95] or induce wound healing [96]. In mammalians, AMPs play role in transition to the adaptive immune response by chemotactic actions for monocytes [97] and T-cells [98]. These peptides also help dendritic cell development with polarizing effects [99]. Next to the orchestration of many parts of the immune responses, they directly affect the infectious agents invading and harming the organisms.

Generally, AMPs are able to act against a broad spectrum of organisms. The antibacterial activity has been the best studied application so far but despite that, the exact mechanism of action is still not completely known. It had been considered that the peptides targeted the cell membrane [82] but there were also some intracellular actions observed, after translocation to the cytoplasm, for example interference with cell wall synthesis, inhibition of protein synthesis or metabolic pathways, interference with nucleic acids or cytokinesis interruption [59].

The activity of AMPs consists of interaction and binding of the peptides with plasma membrane and its permeabilization. The initial association is supposed to be non-specific electrostatic interaction between the cationic peptide and the anionic polar groups of target membrane. On Gram-negative bacteria, the AMPs interact with the lipopolysaccharides, while

on the Gram-positive bacteria they bind to the lipoteichoic acid on the surface. As already mentioned, AMPs possess amphipathic structures enabling their insertion into the membrane bilayer with process driven by hydrophobic interactions. The permeabilization occurs with penetrating the membrane interface and thus causing membrane depolarization, leakage of essential ions and metabolites, decreasing ATP levels and destroying the electrochemical gradient [77].

There are several models of action modes proposed [51]: The **toroidal model** proposes that the amphipathic peptides (generally α-helixes) are inserted into the lipid bilayer and the hydrophilic regions of peptides and lipid groups interact together forming a pore structure (Fig. 12A). In the case of **carpet model**, the peptides form ion interactions between cationic fragments of peptides and anionic groups of the polar heads from outer leaflet of phospholipids of the membrane. Here, AMPs are not inserted into the hydrophobic core of the bilayer they accumulate as parallel oriented, highly organized structures which cause a disintegration of the membrane (Fig. 12B). The mechanism of the **barrel-stave model** involves oligomerization of the peptides, which form transmembrane channels or pores with the hydrophilic residues facing the lumen of the channel. These pores are much smaller than those in toroidal model (Fig. 12C). The newest model is **aggregate channel model**. Here, the peptides just increase the membrane permeability but it is not so high to cause cell death. It was shown, that AMPs competitively displace divalent cations on the surface (Mg^{2+} or Ca^{2+}) and destabilize the bilayer by disruption with lipid-peptide specific domains.

In comparison with current antibiotics, it is considered that the more rapid and less specific mechanisms of action of AMPs lower the probability and possibility in general to acquire resistance, which is the most emergence goal of antimicrobials. AMPs present an attractive tool because the current drug resistance is a main problem in antimicrobial therapy which urgently needs its solution. AMPs are needed to apply especially to the infections by microorganisms with severe resistance to conventional antimicrobials due to the different mechanism of action.

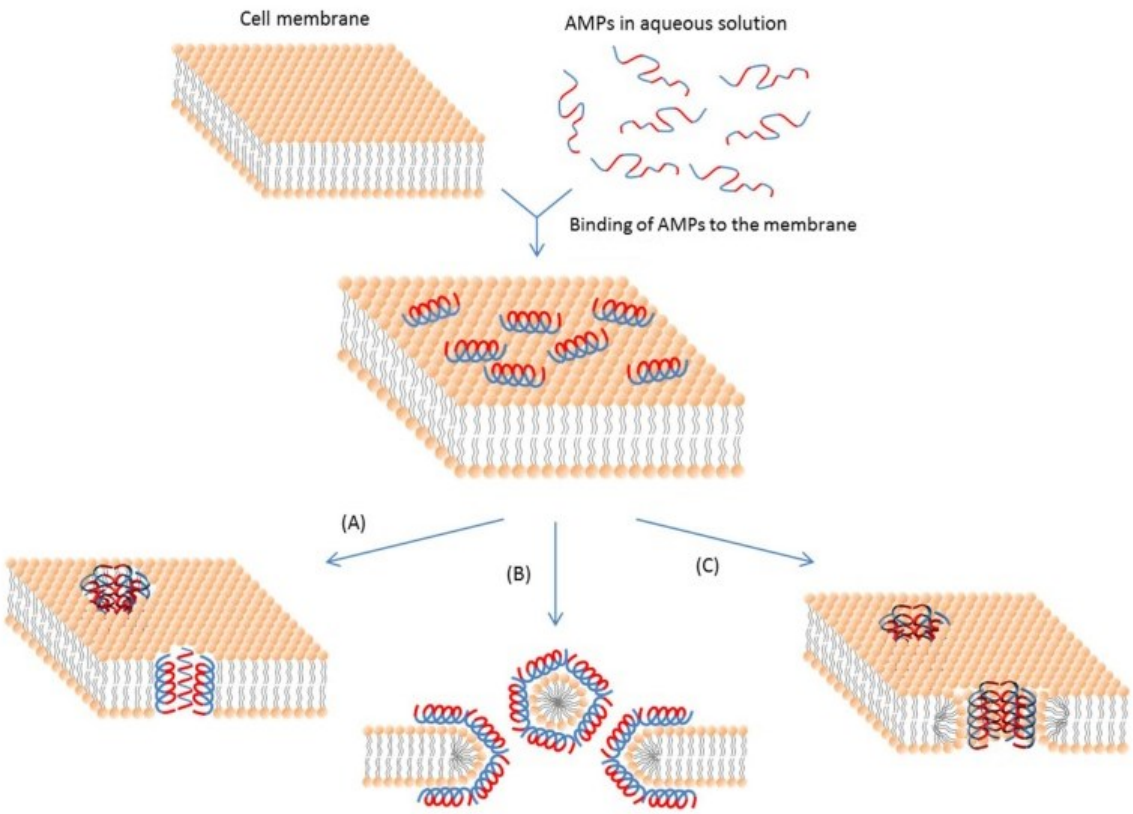


Fig. 12. Three modes of action of AMPs. (A) Toroidal model. (B) Carpet model. (C) Barrel-stave model [51]

1.4.4 Antifungal peptides

Antifungal peptides form one of important groups of AMPs. Antifungal peptides have a significant property in common, which is relatively high appearance of polar and neutral amino acids [59]. Most of them possess the α-helical structures but they do not indicate a sequential or structural homology across the whole group. Such as for antibacterial peptides, definitely, there have been no evident conserved sequence or structural domain established which would determine the antifungal activity [77]. Nevertheless, changes in amino-acid sequence (deletion, substitution or addition of amino acids [100] or conjugation with fatty acids or fusion of different peptides together can lead to notable changes in activity [101].

1.4.4.1 Mode of action of antifungal peptides

The mechanism of action was first described as either cell lysis or interaction with cell wall synthesis or biosynthesis of other essential components of cells [102], e.g. inhibition of chitin synthase (like polyoxins [103] and nikkomycin [104]) or affecting glucan synthesis (like echinocandins [105] and pneumocandins [106]).

In following years, new modes of action, such as production of reactive oxygen species or induction of apoptosis have been identified. Examples of AMPs and the particular sources, structure, target organism and mode of action are listed in Table 1. Still, the exact mechanisms are unknown or controversial sometimes (like histatin 5) and intensively studied. In the case of histatin, the first paper [107] describes the production of ROS in *C. albicans*, while the second paper [108] shows the opposite. However, different techniques for ROS detection were used. The third study [109] suggests the bioenergetic collapse — decrease of mitochondrial ATP synthesis — as the essential mechanism of antifungal effect.

Table 1: Examples of antifungal peptides with the particular sources, structures, targets and modes of action.

Peptide	**Source**	**Structure**	**Target**	**Mode of action**	**Ref.**
Cecropin	Insect	α-helix	*A. fumigatus*	Binds ergosterol/ cholesterol	[110]
Melittin	Insect (bee)	α-helix	*C. albicans*	Induction of apoptosis	[110]
Magainins	Amphibian (frog)	α-helix	*C. albicans*	Lysis	[112]
PMAP-23	Mammal (pig)	α-helix	*C. albicans*	Permeabilization	[113]
Defensin	Mammal	β-sheet	*C. albicans*	Permeabilization and lysis	[114]
Pn-AMP 1	Plant	β-sheet	*C. albicans, S. cerevisiae*	Depolymerase of actin cytoskeleton	[115]
Histatins	Primate	α/β-structured	*C. albicans*	ROS production	[107] [108] [109]
Indolicidin	Bovine	extended	*T. beigelii*	Disrupting of structure of cell membrane	[116]
Lactoferricin	Human, Bovine	α-helix	*C. albicans, S. cerevisiae*	Lysis	[117]
Lactoferrin derived peptide	synthetic	α/β-structured	*C. tropicalis, C. krusei, C. glabrata, C. parapsilosis*	ROS production	[118]
Cathelicidin	synthetic	α-helix	clinical isolated yeast	Lysis	[119]
Drosomycin	Insect	α/β-structured	*F. oxysporum*	Lysis	[120]

1.4.5 Resistance to AMPs

As already mentioned, AMPs are ubiquitous and evolutionarily very ancient. Thus, even though the mode of action of these molecules is diametrically different from the one of conventional antimicrobials, during that long time of development the pathogens had to gain some abilities to resist AMPs. Generally, there are two fundamental strategies, constitutive and inducible resistance, which appear as a continuum and cooperate together [121].

The constitutive (passive) mechanisms of resistance include the actions independent on presence of the peptide and occur also in the absence of it. It comprise inherent mechanisms, such as lack of electrostatic affinity, membrane composition or fluidity of the host cells [121], altered transmembrane potential [122], electrostatic shielding by glycocalyx capsule or biofilm formation [123].

Alternatively, the inducible (adaptive) resistance consists of mechanisms triggered by the AMPs, which are evolved by pathogens to suppress the host defense system. It contains substitution [124] and acylation [125] of phospholipids, modification of lipopolysaccharides (LPS) [126], expressing of repulsive or proteolytic enzymes or efflux pumps [127]. These phenomenons are illustrated in Fig. 13.

Despite of the evolution of those particular resistance mechanisms, it is impossible for pathogens to develop a resistance to all AMPs. However, the reported mechanisms exhibit much less strength than those against conventional antimicrobials and are valid just in a limited number of AMPs.

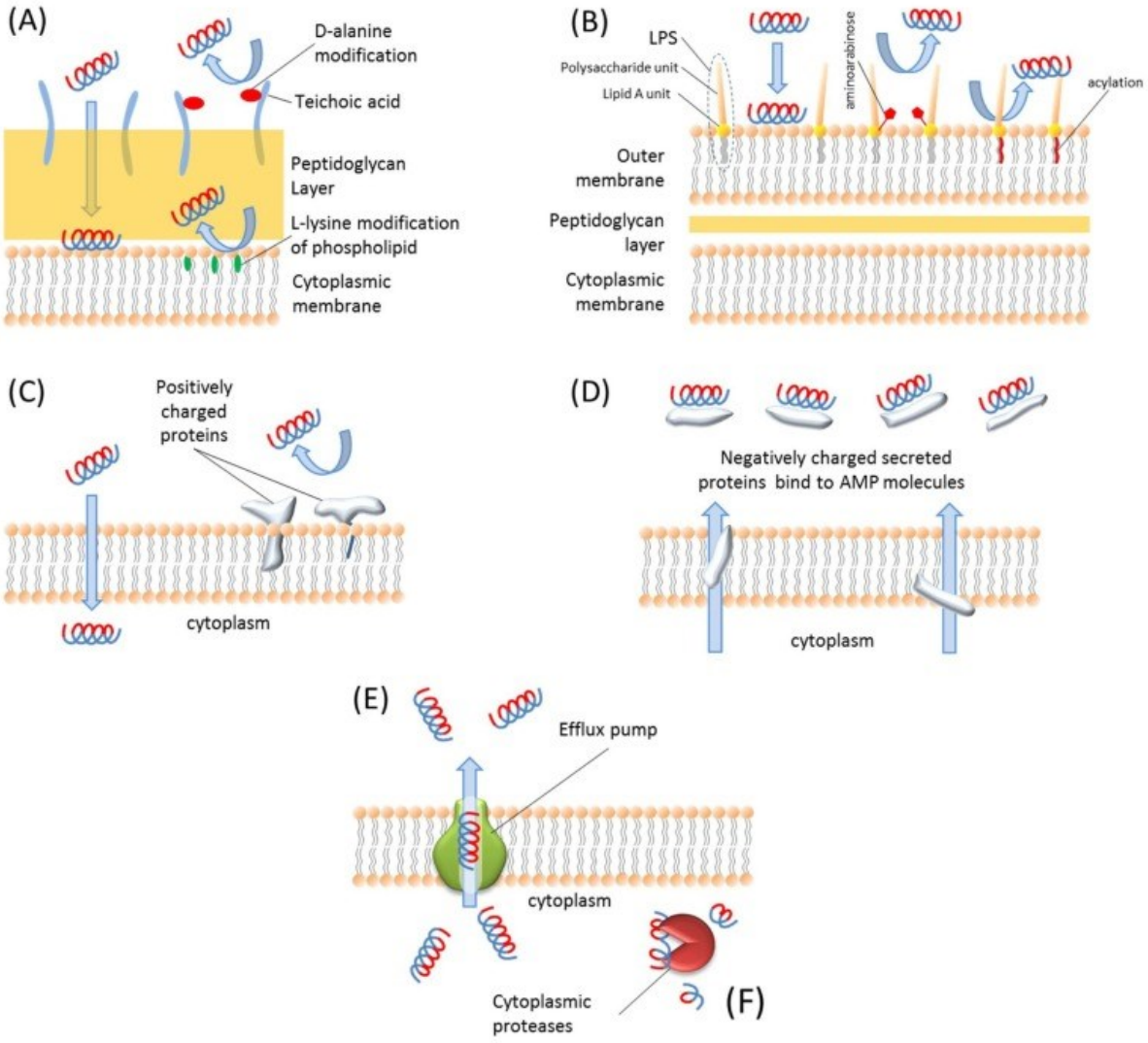


Fig. 13. Schematic representation of AMP resistance mechanisms. (A) Gram-positive bacteria resist AMPs via teichoic acid modification of LPS molecules and L-lysine modification of phospholipids. (B) Gram-negative bacteria resist AMPs by modifying LPS molecules with aminoarabinose or acylation of Lipid A unit of LPS molecules. (C) Bacteria express some positively charged proteins (for example adhesin) and integrate them in the membrane so positive charges repulse each other and bacteria can resist such AMPs. (D) Bacteria produce negatively charged proteins and secrete them into extracellular environment to bind and block AMPs. (E) The intracellular AMPs are extported by efflux pumps. (F) The AMPs inside the cell are degraded by proteases. Taken from [51].

1.4.6 Development and synthesis of AMPs for clinical applications and medical use

Nowadays, the research focused on AMPs is encouraging; These molecules can be potentially used in many areas of our lives, especially in pharmaceutical industry, plant disease control or as food additives. A big advantage of these peptides is their multifunction, such as antimicrobial, antifungal, antiviral or wound healing activities, since many of the diseases are caused by combination of pathogens with different origins so a broad-range treatment is needed [51].

In this field, besides the mentioned new therapeutic approaches to treat infections, AMPs can coat medical devices as bio-disinfectant to inhibit adherence and biofilm formation in immunocompromised patients. Because of the ability to distinguish bacteria, AMPs can act as molecular recognition elements or radiolabeled peptides can also detect infection [128].

AMPs with low toxicity are valuable fungicides for plants, fighting the diseases, harmless for the ecological environment and human health. There were also attempts to produce transgenic crops with integrated genes for broad-spectral peptides [129].

AMPs are also intensively tested for their possible usage as food preservatives with natural origins to replace the chemical ones used until today. Since these molecules can initiate cellular and adaptive immunity, they can potentially act as immunomodulation boosters in food additive. In the last years, the research is heading to the direction of endogenous production to preserve the complete naturalness of AMPs [52].

Like all treatments, AMPs have their limitations and obstacles. It is necessary to mention their cytotoxicity connected with their mechanism of action. AMPs often recognize also membranes of erythrocytes in the host organism and show hemolytic activity against the red blood cells. Another issue comes with the manifestation of AMPs; They are primitive peptide molecules, present almost everywhere in the organisms and mostly they are studied *in vitro* / *in silico* and the main problem occurs in the living system where they interact with the complex environment and the efficiency is not the same. These phenomenons have to be reduced and therefore a lot of effort is put into optimization of the properties of AMPs. There are numerous AMPs isolated from living organisms, naturally acting in their immune systems. According to their structure (primary sequence and secondary structure), there are new

peptides designed. The natural AMPs serve as templates and the novel synthetic ones ideally differ in increased antimicrobial activity and lowered cytotoxicity (especially hemolytic activity). There were many ways found to achieve this aim, with cooperation of various methods in synthesis and bioinformatics [130].

To date, not very clear relationship between the structure and mechanism of action, strength or range of targeted cells has been demonstrated. Thus a prediction of results of AMPs is complicated. But since the length, net charge, hydrophobicity and amphipathicity are crucial for AMPs, semi rational modifying of those features modulates stability, affinity, transport so therefore the effectivity and specificity of the AMPs. A simple change in the primary structure may influence many physicochemical parameters [131]. Several approaches to modify AMPs are used, such as post-translational modifications, e.g. phosphorylation, methylation, glycosylation or addition of D-amino acids [45]. Covalent modifications are also used, e.g. deletion or addition of disulfide bonds is leading to remarkable stability changes [132]. Alteration of amino-acid content belongs to the most important alterations, e.g. higher proline amount results in reduced ability to permeabilize membranes [133]. Last but not least, a helful tool for is homology modelling, which designs new AMPs inspired by known natural peptides and aiming to improve their activity [134].

Various techniques for peptide production were developed, with different adjuvant and carrier systems [135]. Numerous approaches for manufacturing AMPs are currently established, such as biological synthesis, when the peptides are expressed within recombinant systems (mostly *P. pastoris* or *E. coli*). This *in vivo* procedure is the most cost-effective but requires efficient purification and it does not allow incorporating unnatural amino acids [136]. *In vitro* systems with unnatural amino acid tRNAs could solve this problem [137]. Nowadays, chemical synthesis is the major strategy of producing peptide-based drugs. There are three main approaches: Solution-phase [138] is a low cost option with highly pure end-products but long process [135]. Solid-phase [139] is based on coupling of amino acids to an insoluble matrix and is convenient for large-scale production of AMPs [141]. Chemoselective ligation and hybrid synthesis exists but is not used often [141]. Semi-synthesis involves biologically produced peptides further adjusted by chemical and enzymatic methods [142].

1.5 *Galleria mellonella* as a model organism

Various animal models are being used for *in vivo* investigation of *Candida* infections. The mammals as mice are widely spread in research of pathogenicity and virulence. However, in response to its ethical, economical and logistical concerns, new trends are developed nowadays, resulting in so-called "3Rs rules" [143]; There are strong efforts to **replace** the current animal models by different strategies with less bioethical problems, **reduce** the number of animals used in the experiments, **refine** the procedures to decrease the pain. Therefore, instead of rodents as mice or rats new non-conventional models, e.g. insects, are employed to evaluate the relative pathogenicity, virulence and even immune response to the microbial pathogens [144].

Larvae of the wax moth *Galleria mellonella* have many advantages, such as lower expenses for the material, easier manipulation, faster results, and less ethical restrictions. The immune system of this lepidopteran possesses a high level of structural and functional homology with the innate immunity of mammals. The cuticle, analogous to skin, serves as a passive barrier in the first line of defense. The insect body cavity called hemocoel encloses hemolymph which works similar as blood in vertebrates, it is the main site of the immune response, including cellular and humoral elements as well [145]. The insect hemolymph contains cells called hemocytes acting analogically to vertebrates' phagocytes. There have been at least six types of hemocytes identified, responsible for the cellular processes fighting pathogens, such as phagocytosis, nodulization, lysosomal degradation or burst in oxidative metabolism [146]. Humoral immunity in insects is capable of melanisation, hemolymph clotting, wound healing and also AMPs and heat shock proteins production, analogically to vertebrates [147].

Larvae of *G. mellonella* provide an effective and reproducible model for *in vivo* testing of microbial pathogens and antimicrobial compounds, which give results strongly correlating with the data, obtained using mammals [148, 149].

2. Aims of the thesis

The diploma thesis is focused on investigation of AMPs. The main aims are:

- Determination of susceptibility of non-pathogenic *S. cerevisiae* and several pathogenic *Candida* species to conventional antifungal drugs and to AMPs;
- Assessment of the effect of AMPs on membrane potential and viability of yeast cells;
- Comparison of the effect of combination of conventional antifungal drugs and AMPs on *C. glabrata* cells;
- Testing the efficiency of AMPs during *in vivo* *Candida* infection.

3. Materials and Methods

3.1 Materials

3.1.1 Chemicals

Acetic acid	Lach-Ner, Czech Republic
Agar	Dr. Kulich Pharma, Czech Republic
Agarose	Amresco, USA
Citric acid	Lach-Ner, Czech Republic
diS-C_3(3) (3,3'-dipropylthiacarbocyanine iodide)	Sigma-Aldrich, Czech Republic
DMSO (dimethyl sulphoxide) for UV spectroscopy	Sigma-Aldrich, Czech Republic
DNA Ruler markers (λ DNA/HindIII, λ DNA/EcoRI + HindIII, O'GeneRuler 1 kB DNA Ladder)	Thermo-Scientific, Czech Republic
dNTPs (deoxynucleosidtriphosphates)	Sigma-Aldrich, Czech Republic
EDTA (ethylenediaminetetraacetic acid)	Sigma-Aldrich, Czech Republic
Ethanol for UV spectroscopy	Penta, Czech Republic
Glucose	Lach-Ner, Czech Republic
HF buffer	Thermo-Scientific, Czech Republic
Lithium acetate	Sigma-Aldrich, Czech Republic
Mass Ruler DNA Loading Dye	Thermo-Scientific, Czech Republic
MES (2-(N-morpholino)ethanesulfonic acid)	Sigma-Aldrich, Czech Republic
$MgCl_2$ (magnesium chloride)	Thermo-Scientific, Czech Republic
Midori Green	Nippon Genetics, Germany
$Na_2HPO_4 \cdot 12H_2O$ (disodium hydrogen phosphate dodecahydrate)	Penta, Czech Republic
PBS (Phosphate-Buffered Saline)	Sigma-Aldrich, Czech Republic
PCR Ultra H_2O	Top-Bio, Czech Republic
Phusion high-fidelity DNA polymerase	Thermo-Scientific, Czech Republic
PPP MasterMix	Top-Bio, Czech Republic
SDS (sodium dodecyl sulfate)	Sigma-Aldrich, Czech Republic

Sodium chloride solution, 0.9%	Sigma-Aldrich, Czech Republic
Triethanolamine	Sigma-Aldrich, Czech Republic
Tris base	Sigma-Aldrich, Czech Republic
YNB (Yeast Nitrogen Base)	Becton Dickinson, Czech Republic
YPD (Yeast extract Peptone Dextrose) Broth	ForMedium, UK
YPD agar	ForMedium, UK

All chemicals were in high quality, at least grade p. a.

Antifungal drugs:

Amphotericin B	Sigma-Aldrich, Czech Republic
Clotrimazole	Sigma-Aldrich, Czech Republic
Crystal violet	Sigma-Aldrich, Czech Republic
Cycloheximide	Sigma-Aldrich, Czech Republic
Fluconazole	Fresenius Kabi, Czech Republic
5-fluorocytosine	Sigma-Aldrich, Czech Republic
Itraconazole	Sigma-Aldrich, Czech Republic
Ketoconazole	MP Biomedicals, USA
NQO (4-nitroquinoline N-oxide)	Sigma-Aldrich, Czech Republic
Terbinafine	Sigma-Aldrich, Czech Republic

The used antimicrobial peptides (AMPs) are listed in Table 2. The peptides were synthesized by the group of Dr. Václav Čeřovský at the Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences. The peptides were dissolved in sterile 0.9% NaCl solution as 1 mM stock solutions.

The list of used primers for PCRs is shown in Table 3. The primers were ordered from Sigma-Aldrich, Czech Republic, and dissolved in nuclease free water as 100 μM stock solutions.

Table 2: Used AMPs

Peptide	**Sequence***	**Reference**
peptide I	GKWMKLLKKILK-NH_2	[150]
peptide VI	GKWVKLLKKILK-NH_2	[150]
peptide VIII	GKWMKL*l*KKILK-NH_2	[150]
VD8	GKWMKLLK*k*ILK-NH_2	
HYL-3	GIMSSLMKKLAAHIKK-NH_2	[151]
HYL-10	GIMSSLMKKLAKIIKK-NH_2	[151]
HYL-19	GILSSWLKKLKKIIAK-NH_2	[151]
HYL-23	GILSSLLKKWKKIIAK-NH_2	[151]

*D-Amino acids are shown in *italics*. Halictines are shown in blue. Hylanines are shown in orange.

Table 3: Primers for PCR

Oligonucelotide	**Sequence 5'- 3'**
CaCDR1-F1	GGTAGGGATCGGATAGTGG
CaCDR1-R1	CATGAGTGAAAGTTCTGGC
CaCDR1-F2	GGTGGTGAAAGGAAAAGAG
CaCDR1-R2	CCAATCCTTAACCCAGCAC
CaCDR2-F1	CCACAGTTTTGTTGACAAG
CaCDR2-R1	CAACCCCTAATTTCGATGGC
CaCDR2-F2	GATCATTTATGCTGGGTTTG
CaCDR2-R2	CGACTACTACTACAACAACC
CaMDR1-F1	GCAAGAATCAGACACCAAAC
CaMDR1-R1	GCTACGACTCTTCCAATACC
CaMDR1-F2	CATTATTTGGTGCTCCTTTG
CaMDR1-R2	GGTTATCCGTGTTCTATCTC

3.1.2 Cultivation media and buffers

YPD agar:	YPD medium, 2% agar
top YPD agar:	YPD medium, 1% agar
YNB medium:	0.67% yeast nitrogen base without amino acids, 2% glucose
MES buffer:	10 mM MES, pH 3 – 8 adjusted with triethanolamine
CP buffer:	10 mM Na_2HPO_4 pH 6.0 or
	set of buffers prepared according to Table 4
TAE buffer:	40 mM Tris, 20 mM acetic acid, 1 mM EDTA

Table 4: Volumes of disodium hydrogen phosphate and citric acid solutions for preparation of 100 ml of CP buffer of the final pH values

Final pH	**0.2 M Na_2HPO_4 [ml]**	**0.1 M citric acid [ml]**
pH 3	20.55	79.45
pH 4	38.55	61.45
pH 5	51.50	48.50
pH 6	63.15	36.85
pH 7	82.35	17.65
pH 8	97.25	2.75

3.1.3 Instrumentation

Analytical scales CPA225D	Sartorius, Germany
Autoclave 2540 L	Tuttnauer, Slovakia
Camera EOS1300D	Canon, UK
Centrifuge Universal 320R	Hettich, Germany
Flow box SafeFAST Classic2012	Faster, Italy
Gas burner Fuego	WLD-Tec, Germany
Hamilton syringe	Hamilton, Romania

Horizontal electrophoresis MultiSUB midi	Cleaver Scientific, UK
Incubator for 30 °C CLN15	Pol-Eko Aparatura, Poland
Incubator for 37 °C IN110	Memmert, Germany
Magnetic Stirrer RCT basic	IKA, Germany
Microplate reader ELx808	BioTek Instruments, USA
Microscope DM500 with camera EC3	Leica, Germany
PC1 spectrofluorimeter	ISS, USA
pH meter 3510	Jenway, Austria
Power supply for electrophoresis Power Pro	Cleaver Scientific, UK
Replica plater	Sigma-Aldrich, Czech Republic
Scales Compact 440	Kern, UK
Set of pipets	Gilson, USA
Shaking incubator NB-205	N-BIOTEK, Korea
Spectrophotometer Ultraspec 10	Amersham Biosciences, UK
Thermocycler Mastercycler Pro	Eppendorf, Czech Republic
UV transiluminator PhotoDoc-It	UVP, Germany
Vortex mixer VX-200	Labnet International, USA

3.1.4 Yeast strains

The list of used yeast species and the particular strains with their genotypes or other characteristics is shown in Table 5.

Table 5: Yeast strains and their characteristics

Species	**Strain**	**Genotype**	**Source**
Candida albicans	SC5314	wild type	[152]
	CAF2-1	*Δura3::imm434/URA3*	[153]
	DSY448	*Δcdr1::hisG-URA3-hisG/ Δcdr1::hisG*	[153]
	DSY465	*Δmdr1::hisG-URA3-hisG/ Δmdr1::hisG*	[153]
	DSY468	*Δcdr1::hisG/Δcdr1::hisG, Δmdr1::hisG-URA3-hisG/ Δmdr1::hisG*	[153]
	DSY653	*Δcdr2::hisG-URA3-hisG/ Δcdr2::hisG*	[154]
	DSY654	*Δcdr1::hisG/Δcdr1::hisG, Δcdr2::hisG-URA3- hisG/ Δcdr2::hisG*	[154]
Candida glabrata	ATCC 2001	wild type	ATCC collection
	DSY565	azole-resistant clinical isolate	[155]
Candida dubliniensis	CD36	wild type	[156]
Candida krusei	ATCC 6258	wild type	ATCC collection
Candida parapsilosis	CBS 604	wild type	CBS collection
Candida tropicalis	ATCC 750	wild type	ATCC collection
Saccharomyces cerevisiae	BY4741	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF

3.1.5 Animal models

Galleria mellonella R.J.Mous Livebait V.O.F., The Netherlands

3.2 Methods

3.2.1 Cultivation and preparation of yeast cells for the experiments

All the yeast cell stocks were stored in 30% glycerol at -80 °C. For any further usage, first, small amount of the cells was aseptically transferred by a sterile loop from the frozen stock onto YPD agar and incubated overnight at 30 °C. Afterwards, the liquid YPD medium was inoculated by the yeast culture from the solid medium. The cells were grown aerobically in liquid YPD medium with shaking in Erlenmeyer flasks at 30 °C, and harvested from the early exponential growth phase by centrifugation. For the experiments, the cells were washed twice with distilled water and resuspended in the buffer used for the particular experiment.

3.2.2 Growth assays

3.2.2.1 Estimation of growth curves

The microbial growth is determined by an increase in population, number of cells or the increase in overall mass. In this case, yeast growth was monitored by measuring optical density (OD_{600}) of the culture in time [157]. To compare the resistance of the yeast cells to antifungal drugs, the growth rates of cells in liquid media in the presence or absence of drugs were estimated.

In 96-well plate, 100 µl of liquid YPD or YNB media per well were inoculated with 2 µl of cell suspension of OD_{600} = 1, and then cultivated with shaking in an ELx808 reader at 30°C for 24 h. The OD_{600} was measured at 1 h intervals Growth curves were always estimated at least in duplicates.

3.2.2.2 Plating tests

Spread plate technique is a widely used microbiological procedure for quantification of viable microorganisms in a sample [158].

For the experiments, the cells were diluted in MES or CP buffer (pH 3 – 8) to OD_{600} = 0.2. The cell suspension was incubated with the peptides at room temperature for 15 min, 10 µl of the sample was diluted 100-fold in Eppendorf tubes and aliquots of 15 µl were spread on

YPD agar plates in triplicates. For our purpose, we used pre-sterilized glass beads to spread the cell solution within the Petri dish. The colonies were counted after 1 day of incubation at 30 °C. Control samples without exposure to the peptide were regarded as 100 % cell survival.

3.2.2.3 Drop tests

The drop test enables comparison of growth phenotypes of several strains on one Petri dish. This method is based on spotting small volumes – drops – of cell suspensions in serial dilutions on the smooth surface of the solid media containing different compounds [159].

For the experiment, fresh cells grown on YPD agar were directly resuspended in distilled water and adjusted to the same initial OD_{600} = 1.0. Afterwards, 10-fold serial dilutions were prepared in a 96-well plate, and approximately 3 µl aliquots of each dilution were spotted by a replica plater on the YPD agar media with different concentrations of the antimicrobial drugs. To minimize the use of AMPs' stocks (instead of their addition into agar), cells were treated with peptides in the 96-well plate before their transfer onto the agar plates. Plates were then incubated at 30 °C and photographed daily.

3.2.2.4 Disc diffusion tests

The initial *in vitro* determination of the yeast susceptibility to the certain antimicrobial compound can be performed by an agar disc diffusion test. The test is based on the fact that antimicrobial agents diffuse from filter paper discs placed on the agar plate, pre-inoculated by a yeast culture, to the surroundings creating a logarithmic reduction in antimicrobial concentration with the increasing distance from the paper disc which results in forming zones of inhibition around them with the critical concentration. The yeasts growth occurs at a point where the minimum inhibitory concentration at the zones margins is overpowered by the critical mass of the cells. Hence, this method provides the general information about the activity of the antifungal drugs and allows to evaluate the resistance of the microorganisms [160].

The harvested cells were resuspended in 10 mM citrate-phosphate buffer (pH 6.0). Afterwards, they were transfered into top YPD agar (1%) to OD_{600} = 0.2 and poured onto YPD agar (2%) plates and let solidify. Then, the paper discs were laid on the top of the agar and 2

µl of the drug solutions were spotted onto them. The plates were incubated for 2 days at 30 °C and the inhibition zones were photographed and measured.

3.2.3 Measurement of membrane potential with diS-C_3(3) fluorescence probe

All living cells maintain an intracellular negative membrane potential. The used probe, fluorescent cationic dye diS-C_3(3), accumulates inside of the cells proportionally to their membrane potential and allows thus to monitor the relative membrane potential. The dependence of the fluorescence emission maximum wavelength λ_{max} or intensity I_{max} on the time of staining is measured [161].

For the experiment, the cells were resuspended in MES buffer to the OD_{600} = 0.2. Samples (3 ml) were prepared in the suitable cuvettes. The fluorescent probe was added to the cell suspension from 100 µM stock solution to the final concentration of 20 nM in the beginning of the measurement. Fluorescence emission spectra (λ_{ex} = 531 nm, λ_{em} = 560 – 590 nm) were measured in the PC1 spectrofluorimeter. The antimicrobial drugs were usually added after 10 – 20 min of staining with the probe.

3.2.4 *In vivo* testing of antifungal activity of AMPs against *Candida* species in *Galleria mellonella*

To determine *in vivo* effectivity of the AMPs against pathogenic yeast *Candida glabrata*, the wax moth *Galleria mellonella* was injected with the solutions of both pathogen and drugs. The insect larvae were stored at 4 °C in sawdust, and one day before injection they were transferred in Petri dishes with sawdust to 37 °C, 10 larvae per dish. Before injection, the fresh yeast cells were resuspended in 1 ml PBS and the OD_{600} was measured to calculate the infection dose, OD_{600} = 1.0 corresponded to 1.2×10^7 CFU/ml (colony forming unit / ml). The cell suspension was diluted to inject every individual animal with 10 µl containing 3×10^6 cells. Larvae were infected by the pathogen by an injection with a Hamilton syringe to their proleg. 10 µl of 30 µM peptide solution was injected in the same manner immediately after infection by pathogens. Animals injected with 10 µl PBS and non-injected warms were

considered as controls. The larvae were incubated, again in a Petri dish with sawdust at 37 °C, and the alive larvae were counted daily for 7 days.

3.2.5 Genotype verification

3.2.5.1 Polymerase chain reaction

To verify the genotypes of the *Candida albicans* mutants used in this work, PCR (polymerase chain reaction) was performed, in which the presence of genes of interest was estimated. The gene deletions were originally performed in the laboratory of prof. Dominique Sanglard [153, 154] according to the following restriction maps of the *CDR1*, *CDR2* and *MDR1* alleles in disruption experiments (Fig. 14).

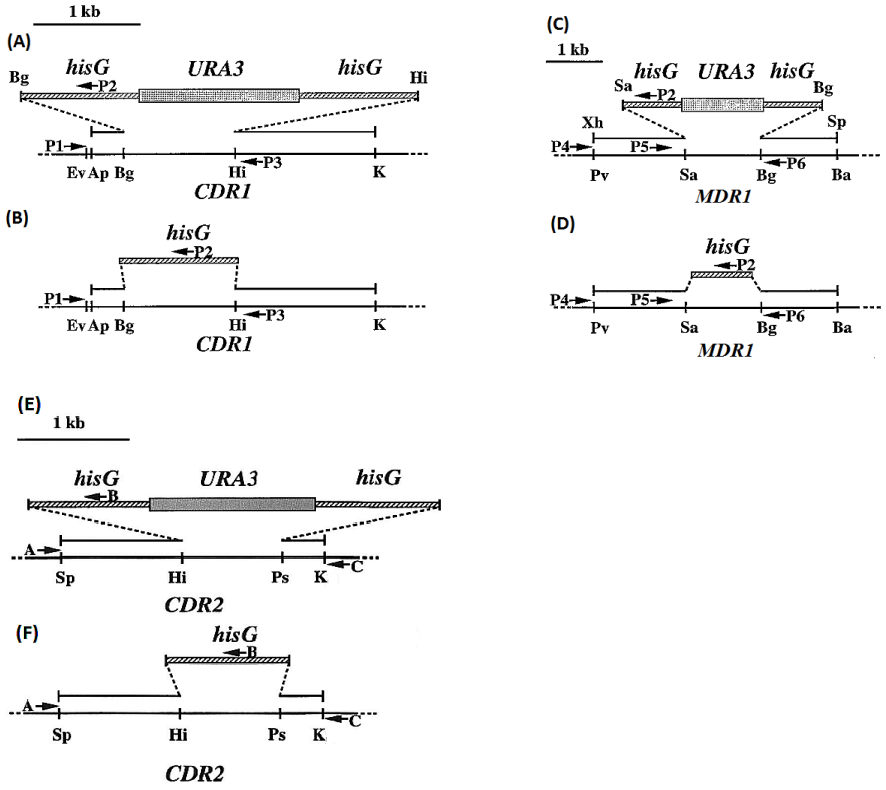


Fig. 14. Representations of the disruption of the *CDR1* (A and B), *MDR1* (C and D) and *CDR2* (E and F) genes before (A, C and E) and after (B, D and F) regeneration of the *ura3* genetic marker by selection with 5-fluoroorotic acid in *C. albicans* strains. The wild-type alleles from *C. albicans* CAF2-1are shown with the corresponding maps of the disrupted alleles. The disrupted alleles are displayed in panels A, C and E match the maps of the linear fragments used for the disruption experiments. Symbols: Ap, *Apa*I; Ba, *Bam*HI; Bg, *Bgl*II; Ev, *Eco*RV; Hi, *Hind*III; K, *Kpn*I; Ps, *Pst*I, Pv, *Pvu*II; Sa, *Sal*I; Sp, *Spe*I; Xh, *Xho*I. The length of *CDR1* is 4506 bp, *CDR2* is 4500 bp, *MDR1* is 1695 bp, *hisG* is 897 bp, *URA3* is 813 bp. Taken from [154, 155].

First, the particular primers for each gene (*CDR1*, *CDR2*, *MDR1*) were designed complementary to the gene sequence from *Candida albicans* genome database (http://www.candidagenome.org/). The sequences of oligonucleotides were assembled and optimized in SeqBuilder software https://www.dnastar.com/t-seqbuilder-pro.aspx] according to the scheme in Fig. 15.

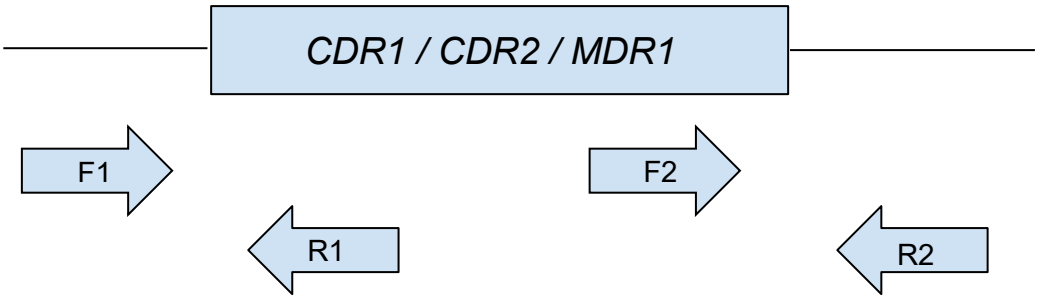


Fig. 15. Schematic localization of oprimers used for the verification of gene disruptions

The length of the expected products of the particular reactions:

CaCDR1-F1—CaCDR1-R1	= 524 bp	
CaCDR1-F2—CaCDR1-R2	= 3.8 kbp	
CaCDR2-F1—CaCDR2-R1	= 845 bp	
CaCDR2-F2—CaCDR2-R2	= 2.7 kbp	
CaMDR1-F1—CaMDR1-R1	= 619 bp	
CaMDR1-F2—CaMDR1-R2	= 491 bp	
CaCDR1-F1—CaCDR1-R2	= 5.1 kbp	original non disrupted gene *CDR1*
CaCDR1-F1—CaCDR1-R2	= 7.8 kbp	disrupted *CDR1* gene by *hisG-URA3-hisG*
CaCDR1-F1—CaCDR1-R2	= 5.0 kbp	disrupted *CDR1* gene by *hisG*
CaCDR2-F1—CaCDR2-R2	= 5.2 kbp	original non disrupted gene *CDR2*
CaCDR2-F1—CaCDR2-R2	= 7.9 kbp	disrupted *CDR2* gene by *hisG-URA3-hisG*
CaCDR2-F1—CaCDR2-R2	= 5.1 kbp	disrupted *CDR2* gene by *hisG*
CaMDR1-F1—CaMDR1-R2	= 2.2 kbp	original non disrupted gene *MDR1*
CaMDR1-F1—CaMDR1-R2	= 4.4 kbp	disrupted *MDR1* gene by *hisG-URA3-hisG*
CaMDR1-F1—CaMDR1-R2	= 1.6 kbp	disrupted *MDR1* gene by *hisG*

Single colonies of yeast strains growing on YPD agar were directly picked from the plates and resuspended in 100 µl of 200 mM lithium acetate, 1% SDS solution in Eppendorf minitubes. Afterwards, the suspension was incubated at 70 °C for 5 min, then 300 µl of 96% ethanol was added and the sample was spinned down. The pellet was washed with 70% ethanol and dissolved in 100 µl nuclease-free water. The cell debris was then spinned down and the supernatant containing DNA was used for the experiment.

Depending on the length of the PCR products, different reaction mixtures were prepared. For fragments shorter than 1 kb, PPP Master Mix was used and for products longer than 1 kb, Phusion polymerase was used. The following reaction mixtures were used and the particular settings of PCR are shown in table 6.

Reaction mixture 1:	PPP Master Mix	6 µl
	DNA template	1 µl
	F primer (10 µM)	0.5 µl
	R primer (10 µM)	0.5 µl
	H_2O	4 µl
Reaction mixture 2:	Phusion polymerase (2 U/µl)	0.2 µl
	DNA template	1 µl
	F primer (10 µM)	1 µl
	R primer (10 µM)	1 µl
	dNTPs (10 mM)	0.4 µl
	HF buffer 5×	4 µl
	$MgCl_2$ (10 µM)	0.4 µl
	H_2O	12 µl

Table 6: Settings of PCR using PPP Master Mix (reaction 1) or Phusion polymerase (reaction 2) mixture

Cycle step	**reaction 1**		**reaction 2**	
	temperature	time	temperature	time
Initial denaturation	94 °C	1 min	98 °C	2 min
Denaturation	94 °C	15 s	98 °C	10 s
Annealing	55 °C	30 s	62 °C	30 s
Extension	72 °C	2 min	72 °C	4 min
Final extension	72 °C	10 min	72 °C	10 min
Cooling	4 °C	forever	4 °C	forever

3.2.5.2 Agarose gel electrophoresis

To see whether the genes of interest are present in the particular yeast genome or not, the amplified PCR products were separated by an agarose gel electrophoresis. By applying the electric field to the negatively charged DNA molecules combined with the loading dye, they migrated through the agarose gel towards the anode and enabled to visualize them.

The 1% agarose gel was prepared in TAE buffer. 10 µl of the PCR product was mixed with 2 µl of 6× loading dye and loaded into the wells in 1% agarose gel, which was stained by Midori Green. The electrophoresis was run in TAE buffer at 100 V for 1 h , and the gel was then photographed under UV light.

4. Results

4.1 Susceptibility of *Candida* species to conventional antifungal drugs

The two most common species causing *Candida* infections, *C. albicans* and *C. glabrata* were compared in order to evaluate the major differences in susceptibilities to conventional antifungal drugs. Representative strains of both species were chosen, laboratory strain *C. albicans* SC5314*, C. glabrata* ATCC 2001 and a clinical isolate DSY565, which is known for its resistance to several antifungal drugs, due to an increased expression of MDR pumps [155].

Disc diffusion tests were performed according to chapter 3.2.2.4 with five chosen antifungal drugs (Fig. 16). According to the presence or absence of growth inhibition zones and their sizes, it can be concluded that amphotericin B had a similar effect on *C. albicans* and *C. glabrata*. Sensitivity of both *C. glabrata* strains to amphotericin B did not differ, as this drug is not a substrate of MDR pumps [26, 32]. In the case of azoles and terbinafine, generally, *C. albicans* was more sensitive to these drugs than *C. glabrata* strains. Moreover, the clinically isolated *C. glabrata* DSY565 was more resistant to clotrimazole, fluconazole and terbinafine in comparison with *C. glabrata* ATCC 2001 strain. These drugs are known to be substrates of multidrug resistance pumps, which are overexpressed in this clinical isolate [39].

Further, in order to estimate whether other *Candida* species resemble by their drug tolerance more *C. glabrata* or *C. albicans*, a detailed growth test involving seven wide spread pathogenic *Candida* species and a laboratory strain of *S. cerevisiae* (described in chapter 3.1.4) in the presence of various antifungal drugs was performed according to chapter 3.2.2.3.

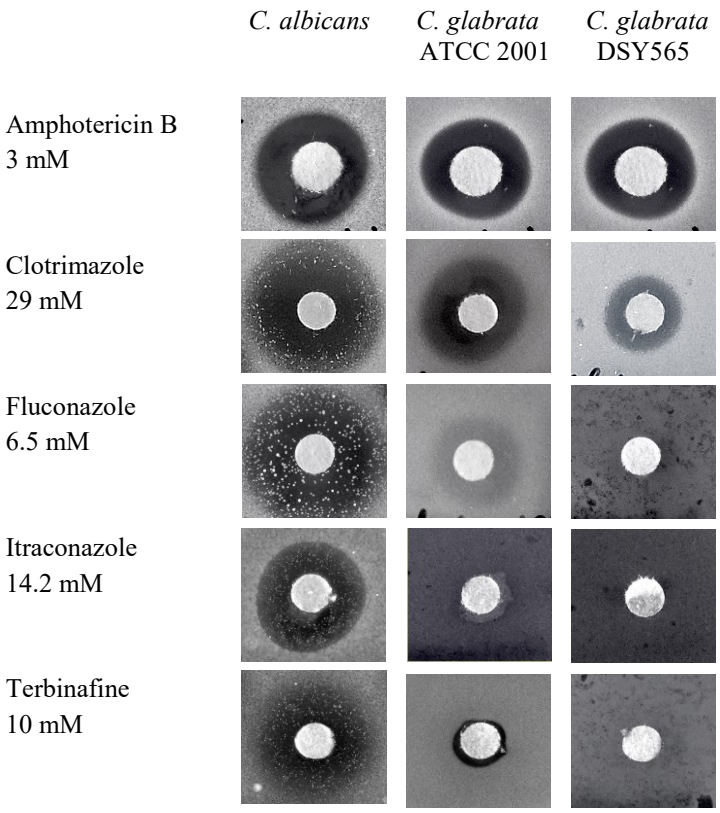


Fig. 16. Growth inhibition zones of *C. albicans*, *C. glabrata* ATCC 2001 and DSY565 cells exposed to amphotericin B, clotrimazole, fluconazole, itraconazole and terbinafine. The enlargement is the same for all the photographs.

The results of the drop tests showed that in general, *C. glabrata* DSY565 was the most resistant strain to azoles from all the species (Fig. 17). Also *C. parapsilosis* and *C. krusei* were able to grow in the presence of 10 μM fluconazole and 0.2 μM ketoconazole better than the other species. *C. krusei* was more sensitive to 0.2 μM itraconazole than to other azole drugs 0.2 μM ketoconazole or 20 μM fluconazole. *C. tropicalis*, *C. dubliniensis* and *C. albicans* were rather sensitive to the tested concentrations of azoles. *C dubliniensis, C. krusei* and *C. tropicalis* were more sensitive to amphotericin B than other *Candida* species and even non-pathogenic *S. cerevisiae.* In contrast, *S. cerevisiae* was the most sensitive species to 5-fuorocytosine and *C. krusei* was the most resistant species to this drug.

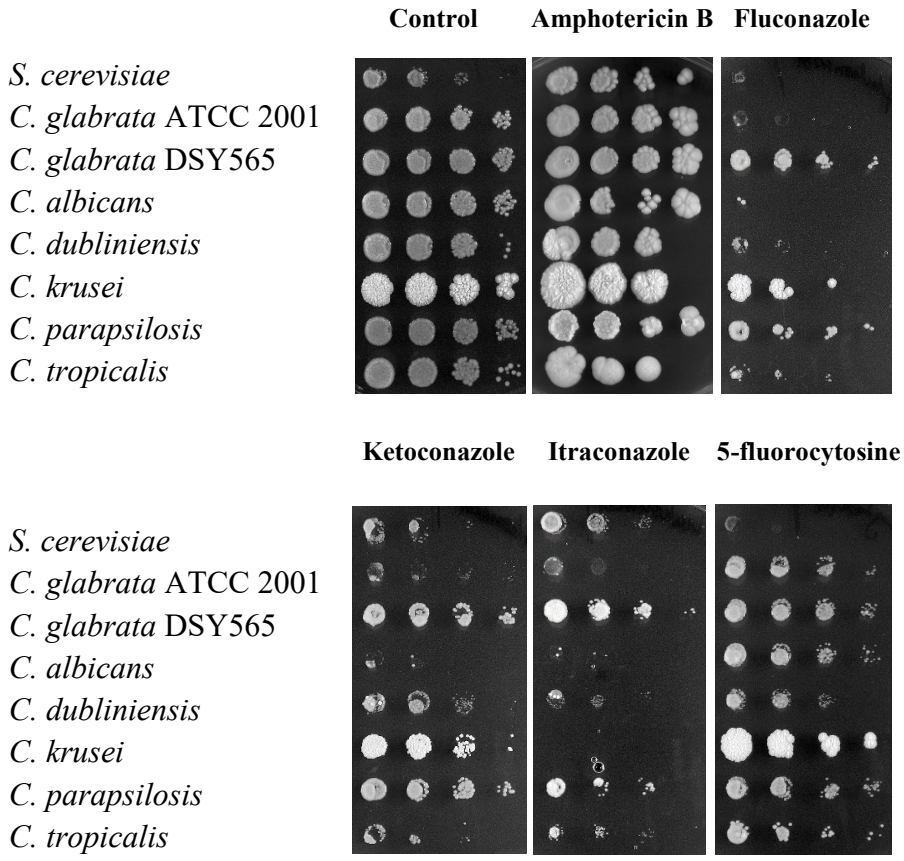


Fig. 17. Growth on solid YPD medium supplemented with 10 μM amphotericin B (recorded after 3 days), 10 μM fluconazole, 0.2 μM ketoconazole, 0.2 μM itraconazole, 20 μM 5-fluorocytosine (recorded after 1 day). The control plate was not supplemented with any antifungal agent (recorded after 1 day).

4.2 The effect of AMPs on *S. cerevisiae* and *Candida* species

4.2.1 Susceptibility of *S. cerevisiae* and *Candida* species to AMPs

For this work, firstly seven AMPs were obtained for investigation of their antifungal activity. The AMPs belonged to two distinct groups; Halictines family (12 aa long peptide chains) comprised the reference peptide I, and derived peptides VI, VIII and VD8; Hylanines family (16 aa long peptide chains) contained the reference HYL-3, and derived peptides HYL-10 and HYL-19. With a view to initially estimate the susceptibility of *S. cerevisiae* and six

pathogenic *Candida* species – in total seven strains (described in more detail in chapter 1.1.2) to those AMPs and to eventually compare the effect of AMPs with conventional antifungal drugs, drop tests were performed. Cell suspensions of seven pathogenic *Candida* strains and one non-pathogenic *S. cerevisiae* strain were incubated with 10 µM AMPs for 15 min, then appropriately diluted and plated in a drop test according to chapter 3.2.2.3.

The results showed that in general, *C. tropicalis* and *C. dubliniensis* were more susceptible to AMPs than other *Candida* species and *S. cerevisiae* (Fig. 18). On the other hand, *C. parapsilosis* possessed a relatively high tolerance to AMPs in a pattern closer to *C. glabrata* strains.

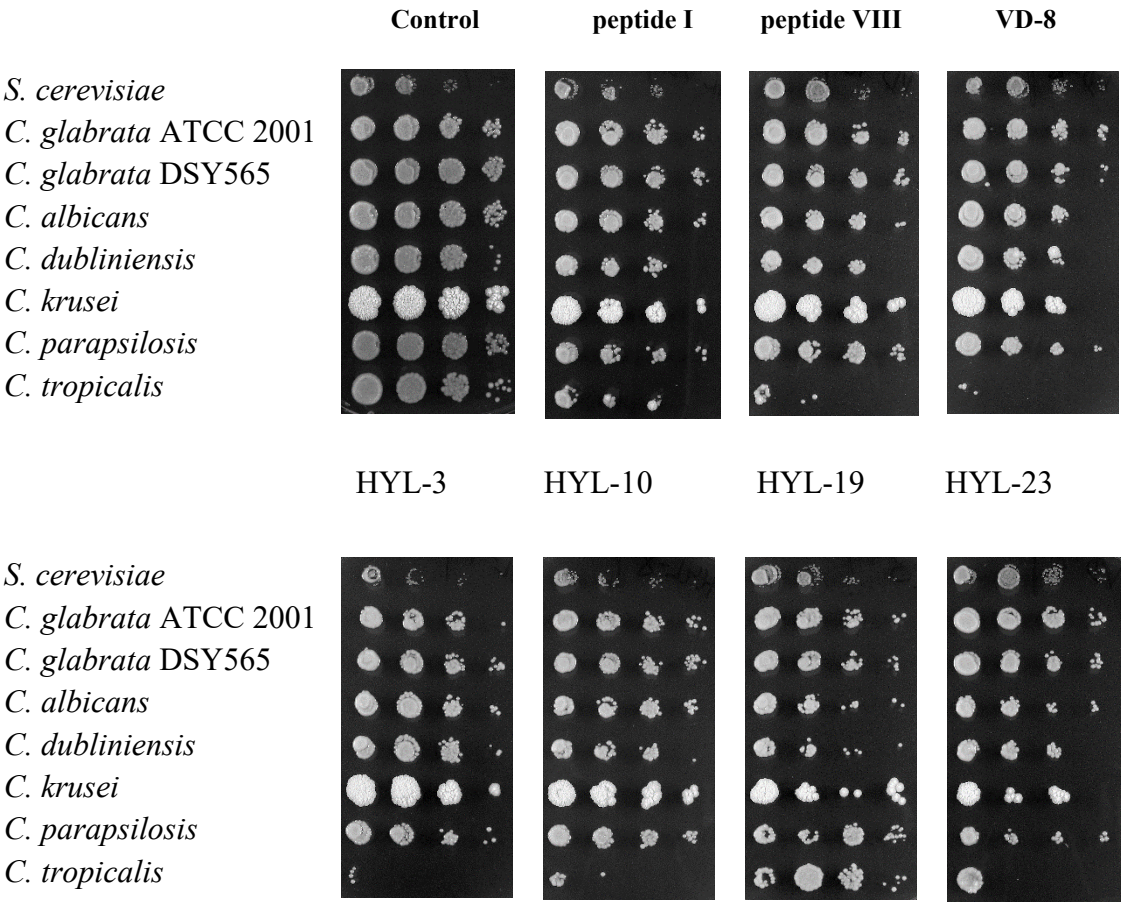


Fig. 18. Growth of cells on solid YPD medium after exposure to 10 µM peptides in distilled water for 15 min. The control plate was not supplemented with any antifungal agents (recorded after 1 day).

4.2.2 Impact of AMPs on membrane potential

To estimate the effect of AMPs on yeast cells, the relative membrane potential and its changes after addition of peptides to the final concentration of 0.2 or 0.5 μM was measured according to chapter 3.2.3 (so called staining curves) and compared with the relative membrane potential of non-treated cells. The changes in membrane potential were recorded as dependence of the fluorescence emission maximum wavelengths λ_{max} (Fig. 19) or the intensity I_{max} (data not shown) on time of staining. AMPs caused hyperpolarization or permeabilization of the plasma membrane, which was reflected by an increase in λ_{max}.

The differences in the efficiencies of the individual AMPs were more distinct when the 0.2 μM concentration was used. The concentration of 0.5 μM caused very strong and similar fast increase in λ_{max}, for all the tested peptides. The experiments were performed with *S. cerevisiae* and six *Candida* species in a similar way.

The staining curves of *S. cerevisiae* and *C. glabrata* after addition of AMPs are shown in Fig. 19, and the results obtained for the rest of tested strains are summarized in Table 7. To quantify the effect of the AMPs, the rates of the changes in membrane potential were evaluated and ordered relatively to each other within every species, as shown in Table 7. Seven AMPs were arranged on a scale, where the highest number (7) indicated the fastest response of the cells, i.e. the most damaging effect, and the lowest (1) the slowest response. The values for each peptide across all the species were added up forming the final number. Then, the AMPs could be ordered according to the final number summarizing the effectivity of individual peptides across the species. Surprisingly, the drug resistant clinical isolate *C. glabrata* DSY565 was highly tolerant to the addition of all the AMPs from hylanines family. The final order of AMPs effectivity was as following: peptide I > HYL-23 > VD-8 > HYL-10 > peptide VIII > HYL-19 > HYL-3

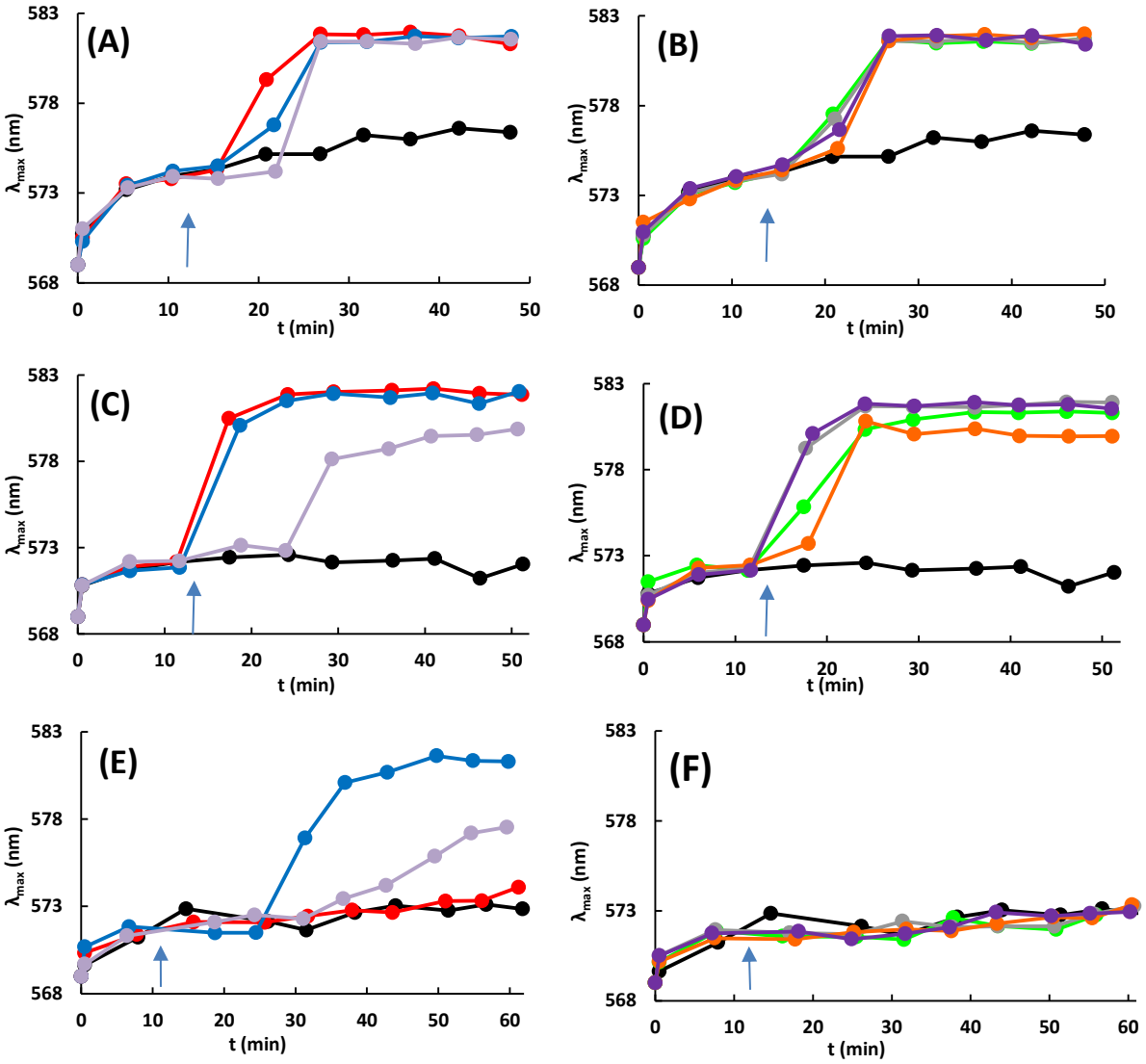


Fig. 19. Relative changes in membrane potential of *S. cerevisiae* (A, B) *C. glabrata* ATCC 2001 (C, D) and *C. glabrata* DSY565 (E, F) after addition of halictines (A, C, E) and hylanines (B, D, F) of final concentration 0.2 µM in time. Control was not treated with any peptide and is displayed in black; peptide I in red; peptide VIII in yellow; VD-8 in blue; HYL-3 in green; HYL-10 in grey; HYL-19 in orange; HYL-23 in purple. The arrows indicate the addition of the AMPs.

Table 7: The relative order of effect of AMPs (0.2 and 0.5 μM) on membrane potential of yeasts

Species	**peptide I**	**peptide VIII**	**VD-8**	**HYL-3**	**HYL-10**	**HYL-19**	**HYL-23**
S. cerevisiae	7	1	4	3	6	2	5
C. glabrata DSY565	5	6	7	0	0	0	0
C. glabrata ATCC 2001	7	1	6	3	4	2	5
C. albicans	3	2	6	1	4	5	7
C. krusei	6	4	1	3	7	2	5
C. dubliniensis	4	5	7	2	1	3	6
C. tropicalis	5	6	2	1	4	3	7
total	37	25	33	13	26	17	35

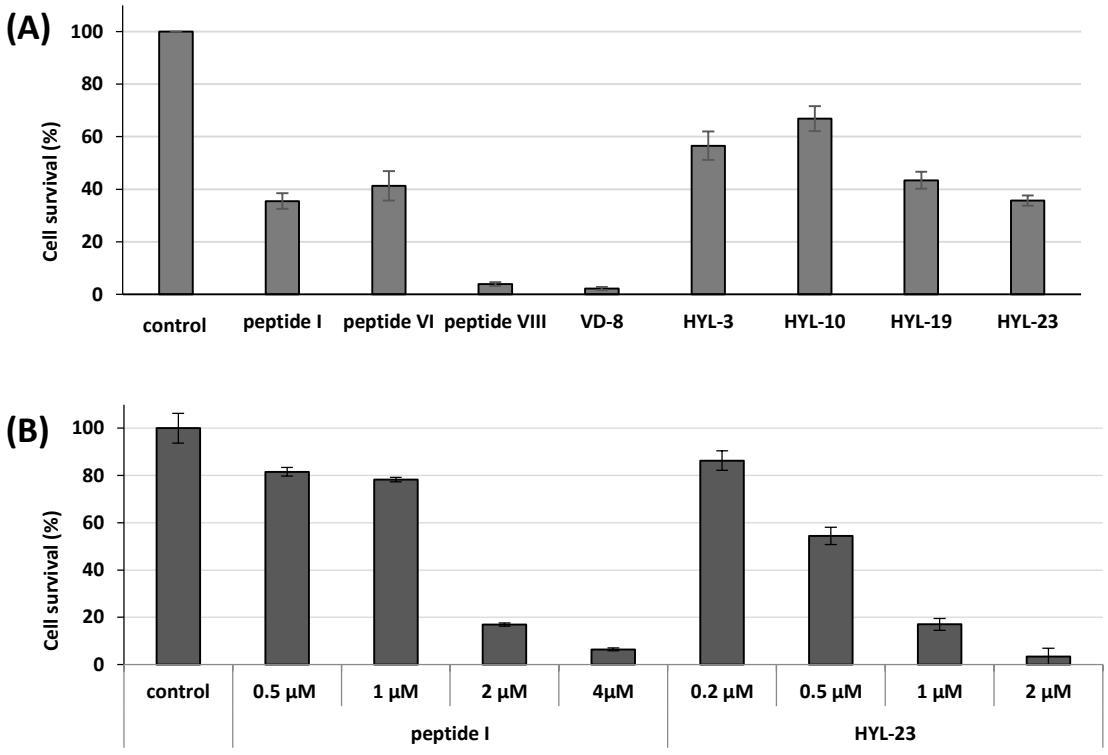
The numbers in the table were assigned for each peptide according to the effect on membrane potential they caused to the particular strain. In total, seven peptides were arranged on a scale within the yeast strain, where the highest number (7) indicated the fastest response of the cells and the lowest (1) signified the slowest response considering both concentrations 0.2 and 0.5 μM of peptides.

4.2.3 Impact of AMPs on viability of *C. glabrata* cells

The mechanism of action of eight AMPs was studied on *C. glabrata* cells. As it was demonstrated in chapter 4.1, *C. glabrata* is more resistant to conventional antifungal drugs than *C. albicans*, therefore new drugs effective against this species are extremely required. In order to evaluate the effect of AMPs on cell viability, plating tests were performed according to chapter 3.2.2.2.

Tested peptides were able to kill *C. glabrata* ATCC 2001 cells in very low concentrations (0.1 μM) in MES buffer at pH 6 (Fig. 21A). These results corresponded to the measurement of changes in relative membrane potential (Fig. 19C, D). As expected, in all cases, higher concentrations of peptides caused lower cell survival of *C. glabrata* ATCC 2001 (Fig. 21B). For this experiment, CP buffer was used instead of MES buffer (Fig. 21A), since

CP buffer was usable in wider range of pH than MES buffer. It is well known that ionic strength has an impact on the antimicrobial activity due to a competition of the positive charges of the cationic AMPs and sodium ions on the negatively charged cytoplasmic membrane [161]. This chart (Fig. 21) comprised not only effect of pH but also Na^+ concentration dependence. CP buffer at pH 4 (Fig. 21B) contained 77.1 mM Na_2HPO_4, the presence of Na^+ ions in such concentration negatively affected the fungicidal activity of cationic peptides similarly to [162, 163]. All the tested peptides possessed bigger killing effect in acidic pH 3 (Fig. 21C), where the concentration of Na^+ was 4-fold lower than at pH 7. To summarize, the effect of AMPs on cell viability after 15 min exposure in MES or CP buffers was dependent on concentration of the AMPs, on pH and Na^+ content in the buffer.



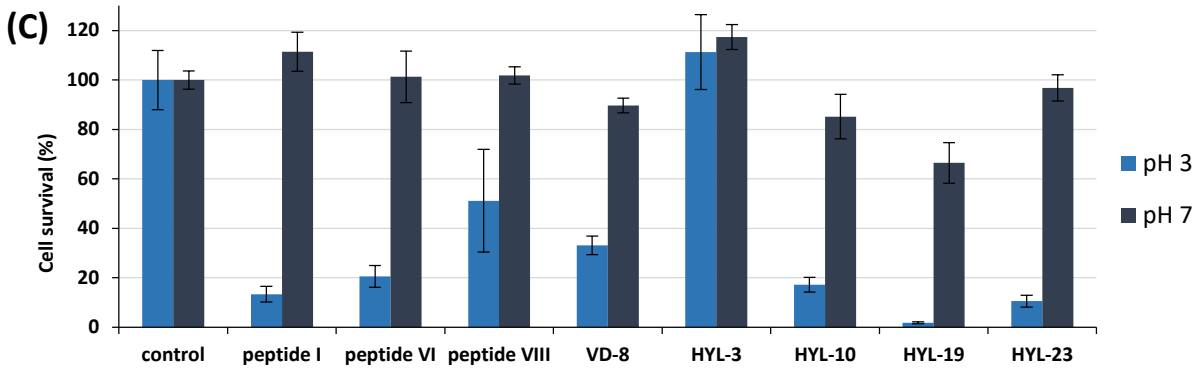


Fig. 21. Killing effect of AMPs shown as survival of *C. glabrata* ATCC 2001cells treated with the AMPs. Untreated cells were considered as 100% survival (control). (A) Cells treated with 0.1 μM peptides in MES buffer at pH 6, (B) cells treated with 0.1 μM peptides in CP buffer at pH 4 (77.1 mM Na_2HPO_4), (C) cells treated with 2 μM peptides in CP buffer at pH 3 (41.1 mM Na_2HPO_4) and pH 7 (164.7 mM Na_2HPO_4).

4.2.4 The effect of combination of AMPs with conventional antifungal drugs on growth of *C. glabrata*

Growth curves were estimated according to chapter 3.2.2.1 for *C. glabrata* ATCC 2001 cells in YPD or YNB medium using antifungals amphotericin B (0.01 – 0.2 μM), clotrimazole (0.1 μM), cycloheximide (10 nM), fluconazole (0.5 – 20 μM), 5-fluorocytosine (0.5 – 20 μM), itraconazole (0.02 – 0.5 μM), ketoconazole (2 μM), terbinafine (2 μM) and eight AMPs in concentrations 0.2 – 100 μM.

Representative growth curves in YPD medium are shown in Fig. 22. No effect of combination of antifungal compounds was observed in YNB media, therefore data are not shown. The time when the growth of control reached its maximum was established. In this time point, the differences between values of OD_{600} of control (untreated) cells and cells which were cultivated in the presence of peptide, conventional antifungal drug or their combination, were clearly visible.

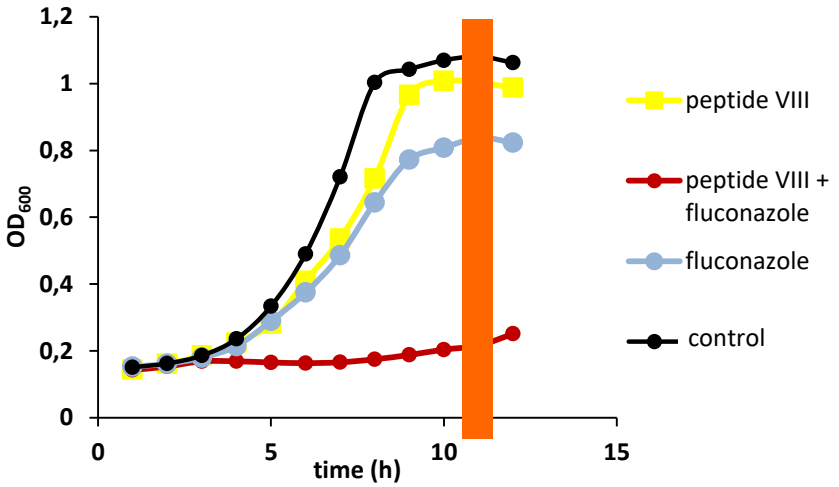


Fig. 22. The growth of *C. glabrata* ATCC 2001 cells in the presence of 30 µM peptide VIII, 10 µM fluconazole and their combination, monitored by change of OD_{600} in time. Untreated cells are considered as control. The values of OD_{600} in selected time (highlighted by a rectangle) were compared.

The values of OD_{600} at the selected time were compared and are presented for 3 selected antifungal drugs in bar charts in Fig. 23. The blue arrows pointed out the cases, in which the peptide had positive antifungal effect in combination with antifungal drug and increased its inhibition effect on *C. glabrata* cells. Whereas all tested peptides were able to improve the killing activity of amphotericin B (Fig. 23C), the effectiveness of combinations with 10 µM fluconazole (Fig. 23A) and 0.2 µM itraconazole (Fig. 23B) were dependent on the individual AMPs. The summary of positive (an increase in inhibition effect of the antifungal drug) or negative (a decrease in inhibition effect of the antifungal drug) effects of all the AMPs combined with all the tested drugs in different concentrations is shown in Table 8 for amphotericin B, in Table 9 for itraconazole, in Table 10 for fluconazole and in Table 11 for 5-fluorocytosine. Different combinations of AMPs were tested in combination with various concentrations of antifungal drugs, all together in 45 combinations. Only the combinations of AMPs and antifungal drugs which possessed positive or negative effect on killing activity of the drug, the other combinations did not lead to any change in inhibition activity of the antifungal drug. Clotrimazole (0.1 µM), cycloheximide (10 µM), ketoconazole (2 µM) and terbinafine (2 µM) are not shown, as no effect of AMPs on the activity of these drugs was observed.

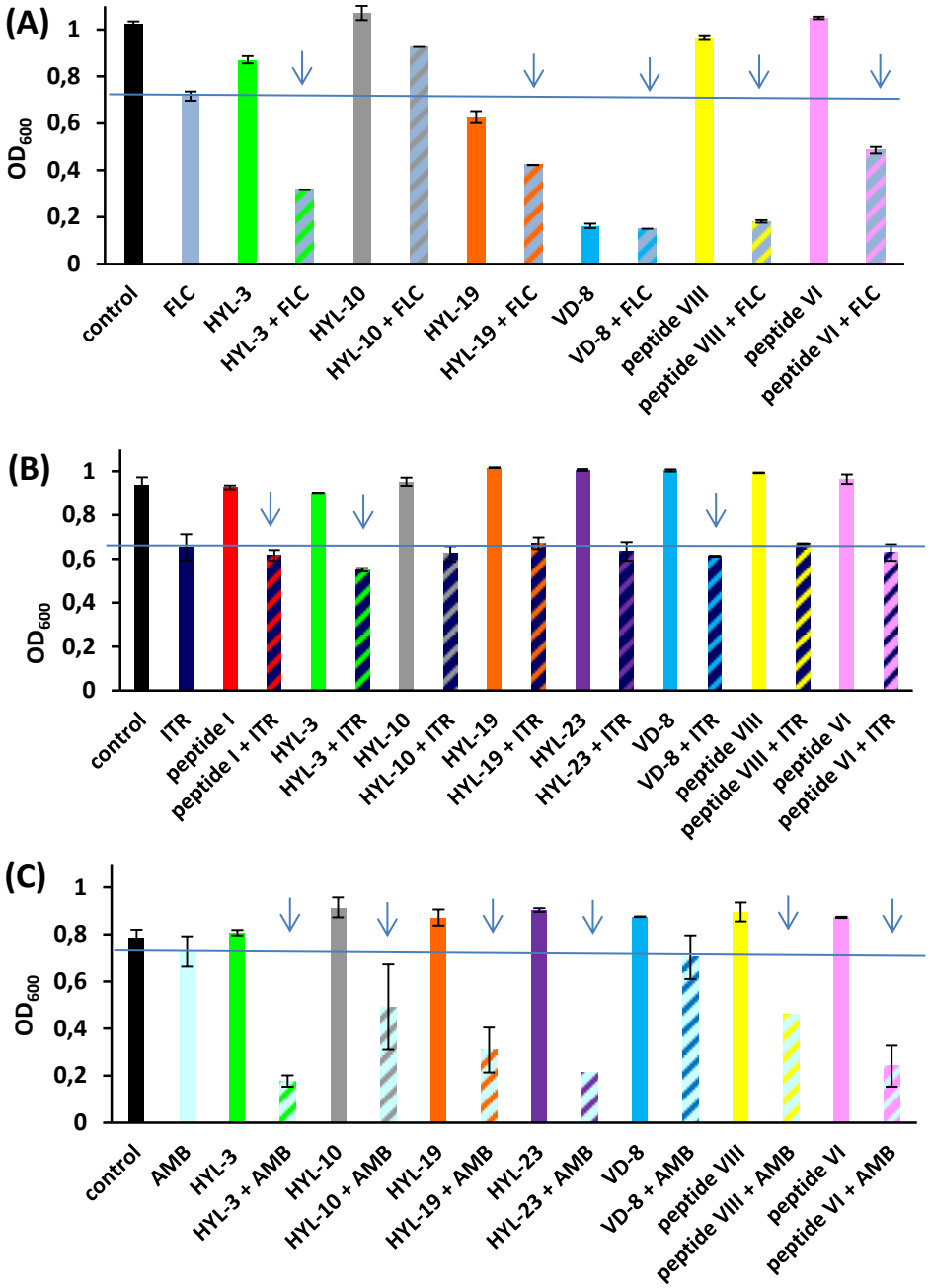


Fig. 23. Relative growth of *C. glabrata* ATCC 2001 cells in YPD medium in the presence of conventional antifungal drugs, AMPs and their combinations. Control was not treated with any drug or peptide. The blue line represents the OD_{600} value of cells treated with drug alone and the arrows point out the cases where the peptide had positive antifungal effect together with the antifungal drug. Cells treated with (A) 10 μM fluconazole (FLC) with 30 μM peptides and (B) 0.2 μM itraconazole (ITR) with 2 μM peptides and (C) 0.1 μM amphotericin B (AMB) with 2 μM peptides estimated as the OD_{600} reached after 12-17 hours of growth of the cell culture.

Table 8: Summary of the positive (+) and negative (-) effects of combinations of AMPs with amphotericin B in various concentrations as a result of growth-curve estimations of *C. glabrata* ATCC 2001 in liquid YPD medium.

Concentration of AMPs	**Peptide**	**Concentration of amphotericin B**	
		0.1 µM	0.01 µM
2 µM	peptide VI	+	
	peptide VIII	+	
	HYL-3	+	
	HYL-10	+	
	HYL-19	+	
	HYL-23	+	
4 µM	peptide VIII	+	
	HYL-3	+	
8 µM	peptide I	+	
	HYL-3	+	
30 µM	peptide VI		+
	HYL-19		+
	HYL-23		-

Table 9: Summary of the positive (+) and negative (-) effects of combinations of AMPs with itraconazole in various concentrations as a result of growth-curve estimations of *C. glabrata* ATCC 2001 in liquid YPD medium.

Concentration of AMPs	**Peptide**	**Concentration of itraconazole**		
		0.2 µM	0.03 µM	0.02 µM
2 µM	peptide I	+		
	VD-8	+		
	HYL-3	+		
8 µM	peptide VIII	+		
	VD-8	+		
30 µM	peptide VI			+
	HYL-3			+
	HYL-10			+
	HYL-19			+
	HYL-23		-	+

Table 10: Summary of the positive (+) and negative (-) effects of combinations of AMPs with fluconazole in various concentrations as a result of growth-curve estimations of *C. glabrata* ATCC 2001 in liquid YPD medium.

Concentration of AMPs	**Peptide**	**Concentration of fluconazole**
		10 µM
2 µM	all	-
30 µM	peptide VI	+
	peptide VIII	+
	VD-8	+
	HYL-3	+
	HYL-19	+

Table 11: Summary of the positive (+) and negative (-) effects of combinations of AMPs with 5-fluorocytosine in various concentrations as a result of growth-curve estimations of *C. glabrata* ATCC 2001 in liquid YPD medium.

Concentration of AMPs	**Peptide**	**5-fluorocytosine**	
		10 µM	0.3 µM
2 µM	all	-	
30 µM	peptide VI		+
	VD-8		+
	HYL-19		+

Efficiency of the combination of antifungal drugs and AMPs was dependent on the concentration ratio: Peptides in lower concentrations (2 – 8 µM) improved the efficiency of amphotericin B and itraconazole. Peptides in higher concentration (30 µM) improved the efficiency of fluconazole.

4.3 *In vivo* testing of AMPs

For the next level of testing of activity of AMPs against *Candida* species, the model organism *Galleria mellonella* was chosen and used according to chapter 3.2.4. To estimate the level of virulence, two strains of *C. glabrata* ATCC 2001 and DSY565 were used (Fig. 26). The insect larvae were infected by the yeast suspensions and alive ones counted daily. The results showed that the clinically isolated strain DSY565 was killing the animals faster than the laboratory strain ATCC 2001. The impact of AMPs on viability of *Galleria* infected by more harmful strain DSY565 was tested by injection of *Candida* cells and 30 µM peptide VD-8 (Fig. 27), which was the most effective peptide in killing of *C. glabrata* ATCC 2001 cells (Fig. 21A and 23A) and also most significantly improved the killing effect of the conventional antifungal drugs (Tables 8 – 10). An AMPs-dependent improvement in survival of larvae was observed. The individuals, which were injected with yeast cells and the peptide were dying more slowly than the ones infected by yeast cells alone. Injection of peptide VD-8 alone did

not negatively affect survival of larvae; It was the same as in the case of larvae injected with pure PBS.

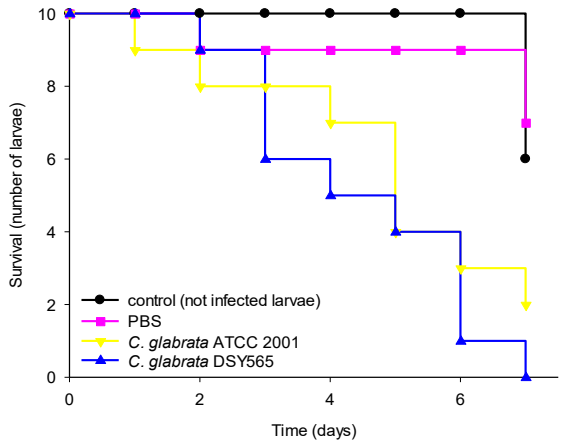


Fig. 26. Comparison of virulence of *C. glabrata* ATCC 2001 and DSY565 strains *in vivo*. Each larvae *Galleria mellonella* was injected with 10 µl of yeast suspension containing 3×10^6 cells. Controls were not injected or injected with PBS.

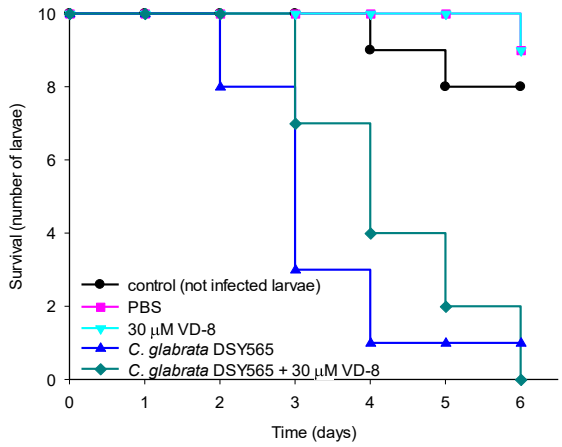


Fig. 27. Viability of larvae *Galleria mellonella* infected by *C. albicans* DSY565 in comparison with larvae injected with the peptide solution alone. Controls were not injected, injected with PBS or injected only with the peptide solution.

4.4 Verification of phenotypes and genotypes of *C. albicans* mutant strains

4.4.1 Phenotype verification

The most common pathogenic yeast species *C. albicans* was intended to be used for experiments with estimating the interaction of AMPs with several MDR pumps. For this purpose, a series of *C. albicans* mutants lacking one or more genes encoding MDR pump, described in more detail in chapter 3.1.4., was obtained from Prof. D. Sanglard [153, 154] As mentioned in chapter 1.3, there are two major families of MDR pumps; Whereas Cdr1 and Cdr2 belong to the ABC transporters family, Mdr1 is a member of the MFS transporters family. The reduced growth of the particular mutant in the presence of a defined inhibitor can be the consequence of an increased accumulation of the drug because of the absence of its specific efflux pump. Therefore, it is possible to assign the inhibitor as putative substrate of MDR pump by observing growth variations between different yeast types on agar plates containing the different inhibitors. Azoles, cycloheximide or terbinafine are specific substrates of the Cdr1 and Mdr1 pumps [165], therefore the mutants carrying the particular deletions are supposed not to grow in the media containing those drugs. Mdr1 pump was previously shown to confer resistance to cycloheximide and NQO as well. Cerulenin is not known as a substrate of any of the studied pumps. For confirmation of presence of appropriate deletions *C. albicans* mutant strains were obtained for comparison with two strains possessing all genes for MDR pumps; SC5314 wild type and its derivative Caf2-1 (Δ*ura3*), which served as a parental strain for the mutants. Drop tests were performed according to chapter 3.2.2.3 with selected antifungal drugs of concentrations according to the source publications and the observed growth phenotypes were compared with the published ones [153, 154].

The growth phenotypes of the DSY448 (*cdr1*Δ) were consistent with the published data (Fig. 24). When the DSY653 strain (*cdr2*Δ) was exposed to the antifungal drugs, no phenotypic effect with respect to drug susceptibility was seen. Only when the *CDR2* gene deletion was present in a *cdr1*Δ background in DSY654 strain, a more severe growth alteration

could be observed. The lack of a specific inhibitor of Cdr2 pump leaded to the closer investigation at the genotype level in the next chapter.

Our growth phenotypes were consistent with the published data except of one particular strain, DSY468, which was supposed to carry double deletion for *cdr1*Δ and *mdr1*Δ. In the published data, the DSY468 strain possessed growth inhibition in plates containing cycloheximide and cerulenin compared with the wild types [153]. However, in our experiments, growth to a greater extent was observed on fluconazole or cycloheximide, which are compounds known to be substrates for the MDR pumps. This fact implied the actual presence and not absence of those pumps, observed in more detail in the next chapter.

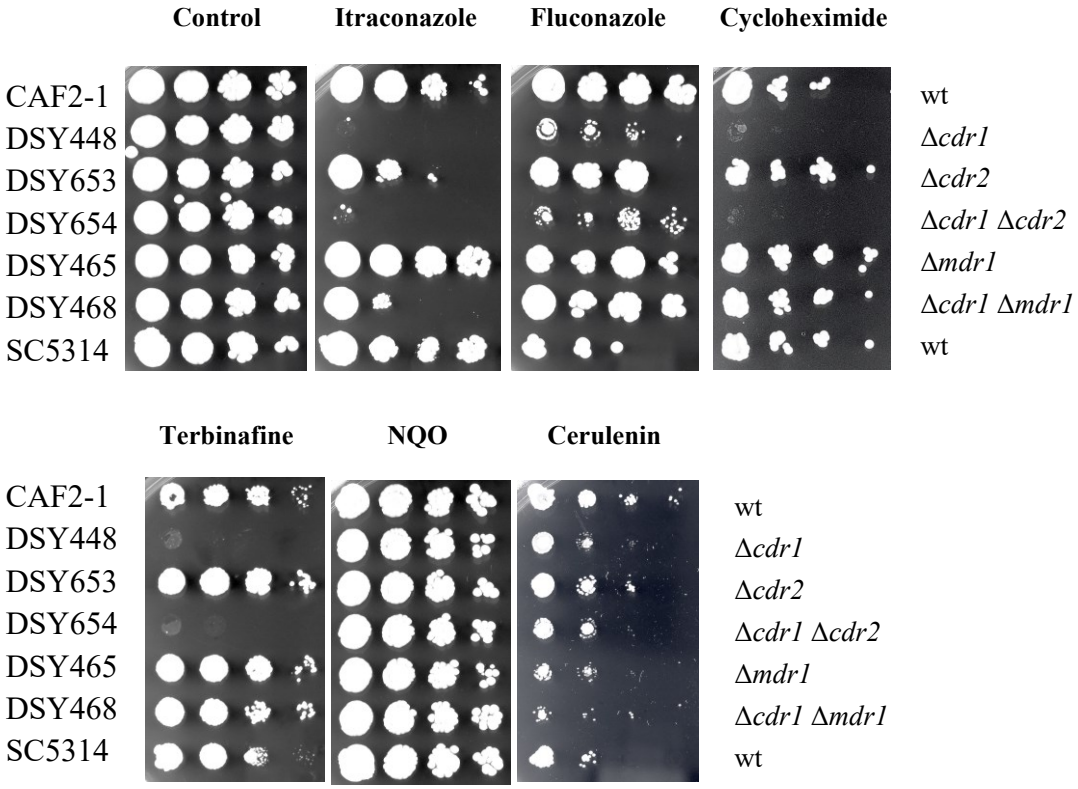


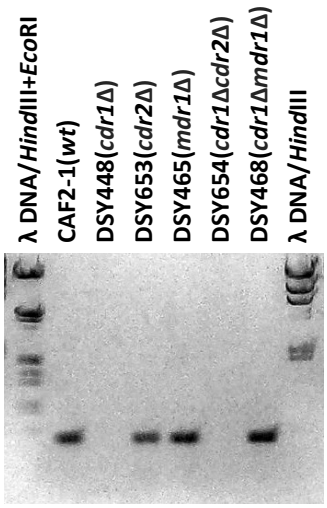
Fig. 24. Drop tests of *C. albicans* mutant strains. The control was not treated with any drug and following concentrations of antifungals present in growth YPD medium were used: itraconazole (0.003μg·ml^{-1}); fluconazole (0.5 μg·ml^{-1}); cycloheximide (400 μg·ml^{-1}); terbinafine (0.25 μg·ml^{-1}); NQO (0.05 μg·ml^{-1}); cerulenin (0.75 μg·ml^{-1}).

4.4.2 Genotype verification

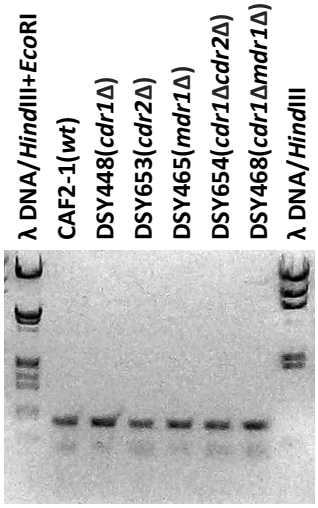
Due to the inconsistent results of growth phenotypes of the mutant strains of *C. albicans* (Fig. 24) with the published papers [153, 154], the presence of particular genes for multidrug resistant pumps was verified by PCR according to chapters 3.2.5.1 and 3.2.5.2. The amplification of the particular DNA fragments revealed the presence or absence of particular fragments of the genes of interest (Fig. 25).

These results suggest that the DSY468 double mutant strain is not carrying the expected genotype *cdr1*Δ*mdr1*Δ. All strains except of DSY468 showed correct genotypes, but for DSY468 double mutant, the PCR analysis revealed that Mdr1 pump is actually present in this strain

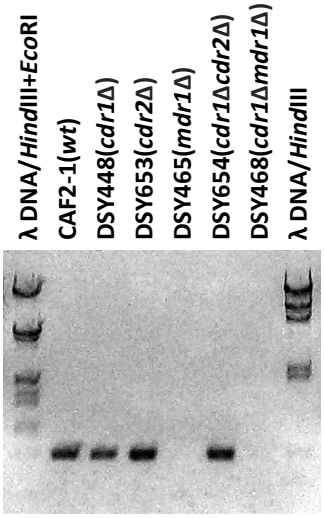
As the double mutant was necessary to characterize the involvement of the particular MDR pumps in resistance to AMPs, the experiments of *C. albicans* mutants and the antifungal peptides were not performed.



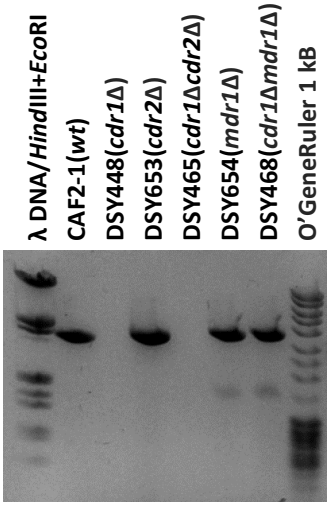
CaCDR1-F1 - CaCDR1-R1(524 bp)



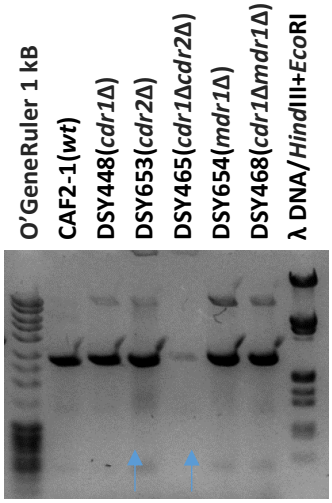
CaCDR2-F1 – CaCDR2-R1(845 bp)



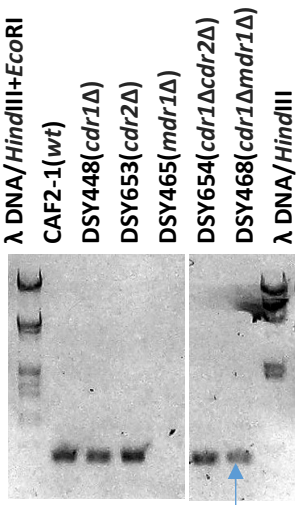
CaMDR1-F1—CaMDR1-R1(619 bp)



CaCDR1-F2—CaCDR1-R2(3.8 kbp)



CaCDR2-F2—CaCDR2-R2(2.7 kbp)



CaMDR1-F2—CaMDR1- R2(491 bp)

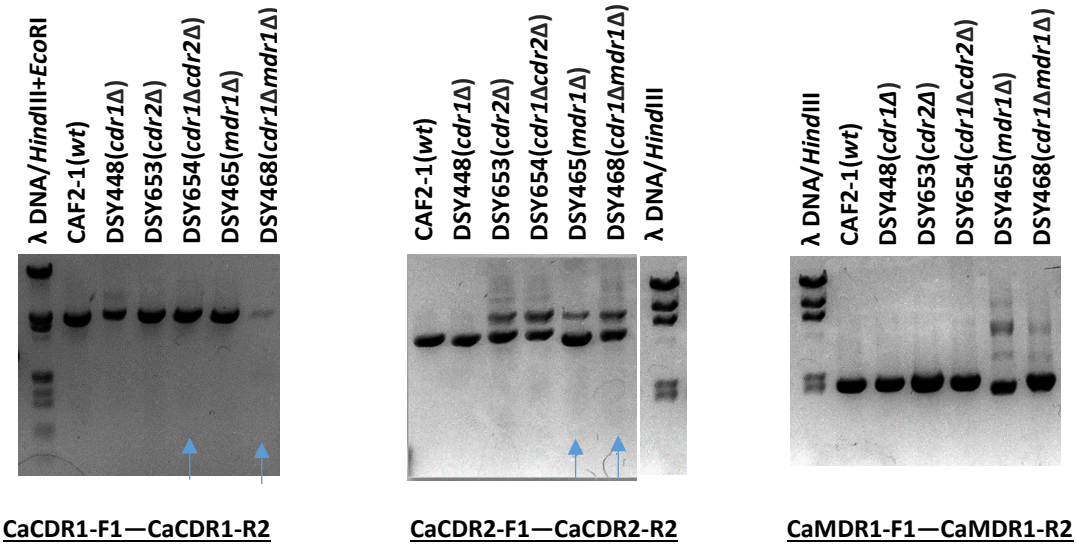


Fig. 25. Photographs of agarose gel electrophoresis of amplified DNA fragments establishing the presence or absence of the genes of interest. The blue arrows indicate unexpected bands which do not correspond with the given genotype of the strain.

According to chapter 3.2.5.1; For *CDR1*: the beginning (F1-R1), the end (F2-R2) and the whole gene from beginning to the end (F1-R2) was missing in expected mutants DSY448 and DSY654 but surprisingly was present in DSY468 where it was supposed to be disrupted. For *CDR2*: The beginning (F1-R1) and the end (F2-R2) of the gene were amplified in all of the tested mutants; In the PCR reaction from the beginning to the end (F1-R2), gene disruption (two bands) was seen in DSY465 and DSY468 where *CDR2* was supposed to be present. Later verification of the sequence of the primers showed, that the oligonucleotides for this particular gene were not designed properly, they were located in the areas of the gene, which were not disrupted,and therefore, in the reactions for detection of *CDR2* fragments (F1-R1 and F2-R2) the disruption did not affect the PCR product, resulting in false positive results. For *MDR1*: the beginning (F1-R1) was missing in expected mutants and the end (F2-R2) of the gene was present in DSY468, where it was supposed to be aborted, but the PCR reaction (F1-R2) confirmed the deletion of this gene in both mutants DSY465 and DSY468 (documented by two bands).

5. Discussion

In this work, non-pathogenic *S. cerevisiae* and six pathogenic *Candida* species – in total seven *Candida* strains – were tested and their sensitivity towards currently used antifungal drugs was estimated. The obtained results revealed that they differ in their tolerance to antifungals. Generally, *C. albicans* is more sensitive to the conventional antifungal drugs than *C. glabrata*, similarly to [5, 10, 12, 14]. Also, it was proven that *C. krusei* and *C. parapsilosis* are more resistant to some azoles than non-pathogenic *S. cerevisiae* or other pathogenic *Candida* species, similarly to [9, 15, 17, 25].

This diploma thesis was focused on investigation of a set of eight antimicrobial peptides (AMPs) targeted towards non-pathogenic *S. cerevisiae* and six pathogenic *Candida* species. The AMPs belonged to two major families, each family of AMPs was derived from one peptide isolated from different wild bee species; Halictines were synthesized according to a reference peptide from eusocial bee *Halictus sexcinctus* [150] and hylanines were synthesized according to a peptide isolated from *Hylaeus signatus* [151].

The most damaging effect on the plasma-membrane level had peptide I from halictines (12 aa) and HYL-23 from hylanines (16 aa). Both AMPs are basic, cationic in neutral pH, their net charge is +5 and 50% of the amino acids are hydrophobic. Those properties allow the AMPs to be electrostatically attracted to the yeast surface and to form the typical amphipathic helixes, as this is their presumable mechanism of action [42, 48, 49, 51], described in more detail in chapter 1.4.3. However, the AMPs are not completely universally effective across all tested *Candida* species; e.g. HYL-10 was the fastest and most effective against *C. krusei*. These differences could be caused by diversity in the composition of the cell walls and the lipid composition of the plasma membranes of those species. In our hands, the use of the fluorescent dye diS-C3(3) proved to be suitable for relative-membrane-potential measurement and estimation of efficiency of the AMPs thus saving the time and material [161].

In this work, also the combination of the AMPs and currently used antifungal drugs was observed. It was shown that the peptide HYL-19 had the most positive influence on fungicidal effect of amphotericin B, itraconazole, fluconazole and 5-fluorocytosine and the peptide VD-8, which also possessed a significantly high killing activity against *C. glabrata*

strains, in addition improved the antifungal effect of itraconazole, fluconazole and 5-fluorocytosine. Overall, there was a strong concentration correlation noticed; The AMPs in lower concentration improved the efficiency of amphotericin B, itraconazole and 5-fluorocytosine, whereas AMPs in relatively higher concentrations improved the efficiency of fluconazole. The results are important for a possible potential application in the future as a desired improvement of combating topical infections [5].

Further testing of AMPs was also performed *in vivo*. Insect model *Galleria mellonella* containing simple immune system in the form of hemolymph was used. It was demonstrated that the selected AMP is not toxic for the organism and moreover it improved larvae survival upon *C. glabrata* infection, which indicates that this AMP was not degraded by the larvae.

Since a significant antibacterial effect of in this work studied AMPs towards gram positive and gram negative bacteria has already been shown [149, 150], the additional antifungal activity towards the six pathogenic *Candida* species suggests a possible potential use of these AMPs in the future, e.g. in combating very common mixed bacterial–yeast infections [10].

6. Conclusions

In this diploma thesis, we focused on investigation of antimicrobial peptides (AMPs). The four main targets were successfully met.

- The susceptibility of non-pathogenic *S. cerevisiae* and several pathogenic *Candida* species to several conventional drugs and AMPs was determined. *C. glabrata* was the most resistant, while *C. tropicalis* and *C. dubliniensis* were the most susceptible species to the studied AMPs.
- The effect of antimicrobial peptides on membrane potential and viability of yeast cells was assessed. The AMPs rapidly permeabilized plasma membrane of yeast cells, which was reflected by a decreased viability of cells. Peptide I, HYL-23 and VD-8 were the most effective ones. The killing potential of AMPs was affected by external pH and concentration of Na^+ ions.
- The positive and negative effects of combination of AMPs with currently used antifungal drugs on resistant *C. glabrata* cells were determined. A strong dependence on the concentration ratio between AMPs and conventional drugs was shown.
- A protective efffect of AMPs during *in vivo C. glabrata* infection was tested. The VD-8 peptide was able to improve survival of *Galleria mellonella* larvae infected by *C. glabrata* cells.

7. References

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Svoluji k zapůjčení této práce pro studijní účely a prosím, aby byla řádně vedena evidence vypůjčovatelů.

Jméno a příjmení s adresou	Číslo OP	Datum vypůjčení	Poznámka